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Reduced IL-2 expression in NOD mice leads to a temporal increase in CD62L^{LO}FoxP3^{+}CD4^{+} T cells with limited suppressor activity

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SUMMARY

IL-2 plays a critical role in the induction and maintenance of FoxP3-expressing regulatory T cells (FoxP3^{+}Treg). Reduced expression of IL-2 is linked to T cell-mediated autoimmune diseases such as Type 1 diabetes (T1D), in which an imbalance between FoxP3^{+}Treg and pathogenic T effectors exists. We investigated the contribution of IL-2 to dysregulation of FoxP3^{+}Treg by comparing wildtype NOD mice with animals congenic for a C57BL/6-derived disease resistant *Il2* allele and in which T cell secretion of IL-2 is increased (NOD.B6Idd3). Whereas NOD mice exhibited a progressive decline in the frequency of CD62L^{HI}FoxP3^{+}Treg due to an increase in CD62L^{LO}FoxP3^{+}Treg, CD62L^{HI}FoxP3^{+}Treg were maintained in the pancreatic lymph nodes and islets of NOD.B6Idd3 mice. Notably, the frequency of proliferating CD62L^{HI}FoxP3^{+}Treg was elevated in the islets of NOD.B6Idd3 versus NOD mice. Increasing levels of IL-2 *in vivo* also resulted in larger numbers of CD62L^{HI}FoxP3^{+}Treg in NOD mice. These results demonstrate that IL-2 influences the suppressor activity of the FoxP3^{+}Treg pool by regulating the balance between CD62L^{LO} and CD62L^{HI} FoxP3^{+}Treg. In NOD mice reduced IL-2 expression leads to an increase in nonsuppressive CD62L^{LO}FoxP3^{+}Treg, which in turn correlates with a pool of CD62L^{HI}FoxP3^{+}Treg with limited proliferation.

Keywords

Immunoregulation; type 1 diabetes; regulatory T cells

INTRODUCTION

The hallmark of Type 1 diabetes (T1D) is the T cell-mediated destruction of the insulin-producing β cells in the pancreatic islets [1-3]. Based on studies in humans and the NOD mouse, a spontaneous model of T1D, the breakdown of β cell-specific tolerance is in part due to defective peripheral immunoregulation within the T cell compartment. Conventional

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T cells in NOD mice for instance, exhibit reduced sensitivity to the suppressive effects of immunoregulatory T cells (Treg) [4, 5]. The loss of function and/or frequency of Treg has also been implicated in the differentiation and expansion of pathogenic type 1 effector T cells specific for β cells [5-7]. Several subsets of Treg with distinct phenotypes and effector functions have been identified [8] including: i) type 2 T effectors which predominantly secrete IL-4, ii) Th3 cells, which primarily secrete IL-4 and TGFβ [9], iii) IL-10 secreting Treg [10], and iv) natural and adaptive $CD4^+CD25^+$ T cells which express the transcription factor Forkhead box P3 ($FoxP3^+$Treg) [11].

$FoxP3^+$Treg are considered to be the most potent subset of Treg, and are characterized by a suppressor function mediated by cell-cell contact-dependent and -independent mechanisms [12]. Humans and mice lacking functional FoxP3 protein develop systemic T cell-mediated autoimmunity [13-15]. $FoxP3^+$Treg suppress T cells through constitutive expression of CTLA-4 and the glucocorticoid-induced TNF receptor (GITR) which block co-stimulatory signals needed for T cell activation [16]. Additionally, $FoxP3^+$Treg elicit suppression through a bystander effect via TGFβ [12, 17], which modulates the function of APC and inhibits production of IFNγ and TNFα by type 1 T effectors [18].

The phenotype of $FoxP3^+$Treg can be further defined based on CD62L expression. For instance, the *in vitro* and/or *in vivo* suppressor function of $CD62L^{HI}FoxP3^+$Treg is superior compared to $CD62L^{LO}FoxP3^+$Treg [7, 19, 20]. Furthermore, $CD62L^{HI}FoxP3^+$Treg from the pancreatic lymph nodes (PLN) or spleen of NOD mice exhibit an enhanced capacity to prevent diabetes in an adoptive transfer model compared to $CD62L^{LO}FoxP3^+$Treg [19]. Increased levels of TGFβ expression contributes to the enhanced suppressor function of $CD62L^{HI}FoxP3^+$Treg versus $CD62L^{LO}FoxP3^+$Treg [7]. $CD62L^{LO}FoxP3^+$Treg are thought to reflect an activated phenotype characterized by increased cycling [21-23]. Importantly, our group and others have previously shown that the frequency of suppressive $CD62L^{HI}FoxP3^+$Treg decline with age in NOD female mice which corresponds with the progression of β cell autoimmunity [7, 24]. The critical events that induce and maintain the frequency of $CD62L^{HI}FoxP3^+$Treg, however, are poorly understood.

Recent studies have demonstrated that IL-2 plays a key role in the maintenance of $FoxP3^+$Treg homeostasis [25, 26]. Mice lacking or having reduced expression of the *Il2* gene develop severe, systemic autoimmunity due to the reduction of $FoxP3^+$Treg [27, 28]. Furthermore, Sakaguchi *et al.* showed that diabetes is exacerbated in NOD mice when treated with a neutralizing Ab specific for IL-2 at an early age [29]. Also, IL-2 in combination with TGFβ is important for the differentiation of naïve $CD4^+$ T cells into adaptive $FoxP3^+$Treg *in vitro* [30, 31].

More than 20 chromosomal loci, termed insulin-dependent diabetes (*Idd*) regions, are associated with T1D susceptibility and resistance [32, 33]. While no one gene is sufficient for the development of diabetes, the combined effects of susceptibility genes influence the progression of β cell autoimmunity [32, 33]. NOD mice congenic for the *Idd3* locus derived from diabetes-resistant mouse strains exhibit a reduced incidence and delayed onset of T1D [34-37]. *Idd3* contains genes encoding immunoregulatory molecules including IL-2 and IL-21 [34-37]. The NOD *Idd3* locus has been associated with reduced IL-2 expression by T cells and an aberrant $FoxP3^+$Treg pool [37, 38]. These findings suggest that T1D is influenced by dysregulation of IL-2 expression, which leads to reduced $FoxP3^+$Treg frequency and/or function found in NOD mice. In the current study, NOD mice congenic for a resistant *Idd3* interval derived from C57BL/6 (B6) mice (NOD.B6Idd3) were used to further define the role of IL-2 in regulating the peripheral $FoxP3^+$Treg pool. We present evidence that reduced IL-2 expression leads to temporal dysregulation of the ratio between suppressor-deficient $CD62L^{LO}FoxP3^+$Treg and suppressor-competent

CD62L^{HI}FoxP3^{+}Treg, resulting in a pool of FoxP3^{+}Treg insufficient to regulate β cell autoimmunity.

RESULTS

An age-dependent decline in CD62L^{HI}FoxP3^{+}Treg is detected in NOD but not NOD.B6Idd3 mice

Studies have demonstrated that *Idd3* in NOD mice contributes to the progression of β cell autoimmunity by influencing the pool of FoxP3^{+}Treg [37, 38]. To further study the effect(s) of *Idd3* on FoxP3^{+}Treg, NOD.B6Idd3 mice congenic for an ~17 Mb interval derived from the B6 genotype were employed (Supplementary Table 1). This line of NOD.B6Idd3 female mice exhibited a reduced frequency of diabetes and insulitis relative to NOD female mice (Fig. 1), similar to other NOD mouse lines congenic for a resistant *Idd3* locus [37-39]. Consistent with previous findings [38] naïve CD4^{+} T cells isolated from the spleen of NOD.B6Idd3 mice exhibited increased IL-2 secretion upon *in vitro* stimulation relative to NOD CD4^{+} T cells (Supplementary Fig. 1). To determine the influence of *Idd3* on FoxP3^{+}Treg, the frequency and number of gated CD4^{+}CD3^{+} T cells expressing FoxP3 and CD25 (Fig. 2A) were assessed in the thymus, spleen, PLN and islets of age-matched NOD and NOD.B6Idd3 female mice via FACS. No difference in the frequency of FoxP3^{+}Treg was detected in the thymus of NOD and NOD.B6Idd3 mice suggesting that thymic development of FoxP3^{+}Treg is unaffected by IL-2 expression levels. On the other hand, an increased frequency and number of FoxP3^{+}Treg was detected in the PLN and spleen of older NOD.B6Idd3 mice relative to age-matched NOD mice (Fig. 2A-C). In addition, the frequency of FoxP3^{+}Treg was significantly increased in the islets of 10 and 16 wk-old NOD.B6Idd3 versus NOD female mice (Fig. 2B). Notably, however, a greater number of FoxP3^{+}Treg was detected in the islets of older NOD mice (Fig. 2C) reflecting increased T cell infiltration of the islets relative to age-matched NOD.B6Idd3 mice. These data demonstrate that the frequency of FoxP3^{+}Treg is increased in the PLN and islets of NOD.B6Idd3 mice compared to NOD mice.

We and others have shown that CD62L^{HI}- versus CD62L^{LO}-expressing FoxP3^{+}Treg exhibit increased suppressor activity [7, 19]. Accordingly, CD62L^{HI}- and CD62L^{LO}-expressing FoxP3^{+}Treg were examined temporally in age-matched NOD.B6Idd3 and NOD female mice. Interestingly, age-dependent differences in the frequency and number of CD62L^{HI}- and CD62L^{LO}-expressing FoxP3^{+}Treg were detected in the PLN and islets of the respective groups of mice. NOD female mice exhibited a temporal decrease in the frequency of CD62L^{HI}FoxP3^{+}Treg and a concomitant increase in CD62L^{LO}FoxP3^{+}Treg in PLN (Fig. 3B). Although the number of CD62L^{HI}FoxP3^{+}Treg progressively increased in the PLN of NOD female mice (5.2×10^4 (4 wks) versus 9.0×10^4 (16 wks)), a greater increase in CD62L^{LO}FoxP3^{+}Treg numbers was detected (6.3×10^4 (4 wks) versus 14.9×10^4 (16 wks)) (Fig. 3C). In the PLN of NOD.B6Idd3 mice, however, the frequency and number of CD62L^{HI}FoxP3^{+}Treg showed no marked change with age, which were increased relative to age-matched NOD females (Fig. 3B,C).

A similar scenario was observed in the islets of NOD and NOD.B6Idd3 female mice. A temporal increase in the frequency of CD62L^{LO}FoxP3^{+}Treg was detected in the islets of NOD female mice which was due to elevated numbers relative to CD62L^{HI}FoxP3^{+}Treg (Fig. 3D,E). Despite a progressive decline, the frequency of CD62L^{HI}FoxP3^{+}Treg in the islets of NOD.B6Idd3 female mice was elevated relative to age-matched NOD female mice (Fig. 3D,E). FACS analysis showed similar levels of CD25, GITR and CTLA-4 surface expression between CD62L^{HI}FoxP3^{+}Treg and CD62L^{LO}FoxP3^{+}Treg infiltrating the islets of either NOD or NOD.B6Idd3 mice (data not shown).

Differences in the proliferative status of $CD62L^{HI}$- versus $CD62L^{LO}$-expressing $FoxP3^{+}$Treg could explain the distinct $FoxP3^{+}$Treg profiles seen in the islets of NOD and NOD.B6Idd3 mice. To investigate this possibility, proliferation of $CD62L^{HI}CD4^{+}CD25^{+}FoxP3^{+}$ and $CD62L^{LO}CD4^{+}CD25^{+}FoxP3^{+}$ T cells was assessed via Ki67 staining in the islets of 12 wk-old NOD and NOD.B6Idd3 female mice. Regardless of the genotype, the frequency of proliferating $CD62L^{LO}CD4^{+}CD25^{+}FoxP3^{+}$ T cells was elevated relative to $CD62L^{HI}CD4^{+}CD25^{+}FoxP3^{+}$ T cells (Fig. 4B). Importantly, however, the frequency of proliferating $CD62L^{HI}CD4^{+}CD25^{+}FoxP3^{+}$ T cells (Fig. 4B) and the ratio of Ki67-staining $CD62L^{HI}CD4^{+}CD25^{+}FoxP3^{+}$ to $CD62L^{LO}CD4^{+}CD25^{+}FoxP3^{+}$ T cells (Fig. 4C) were increased in the islets of NOD.B6Idd3 versus NOD female mice. Together, these results indicate that within the pool of $FoxP3^{+}$Treg a significant shift from $CD62L^{HI}FoxP3^{+}$Treg to $CD62L^{LO}FoxP3^{+}$Treg occurs in the PLN and islets of NOD but to a lesser extent in NOD.B6Idd3 female mice, which correlates with a decreased proliferative status of $CD62L^{HI}FoxP3^{+}$Treg in NOD versus NOD.B6Idd3 mice.

Increased $CD62L^{HI}FoxP3^{+}$Treg enhance the suppressor activity of the pool of $FoxP3^{+}$Treg from NOD.B6Idd3 versus NOD mice

Elevated numbers of $CD62L^{HI}FoxP3^{+}$Treg in NOD.B6Idd3 mice would be expected to enhance suppression of pathogenic T effectors in the respective tissues. Indeed, at 16 wks of age the frequency of insulitis is reduced in 16 wk-old NOD.B6Idd3 versus NOD female mice (Fig. 1B). Consistent with the latter the ratio of $CD62L^{HI}FoxP3^{+}$Treg versus IFNγ-secreting $CD4^{+}$ T cells in the islets and PLN was significantly increased in 16 wkold NOD.B6Idd3 versus NOD female mice (Fig. 5A). The overall frequency of proliferating T cells was reduced in the islets of 16 wk-old NOD.B6Idd3 versus NOD female mice (Fig. 5B). To directly assess the *in vivo* suppressor activity of NOD and NOD.B6Idd3 $FoxP3^{+}$Treg, co-adoptive transfer experiments were carried out. $CD4^{+}CD25^{+}$ T cells were prepared from PLN of 16 wk-old NOD.B6Idd3 or NOD female mice, co-injected with splenocytes from diabetic NOD donors into NOD.*scid* mice, and diabetes monitored. Importantly, the frequency of FoxP3-expressing cells in the pool of sorted $CD4^{+}CD25^{+}$ T cells was similar between NOD and NOD.B6Idd3 donors (72±5% and 75±3, respectively; average of 3 separate experiments). As expected all NOD.*scid* mice receiving diabetogenic splenocytes-alone developed diabetes (Fig. 5C). Similarly, the entire group of NOD.*scid* mice injected with a mixture of diabetogenic splenocytes plus NOD $CD4^{+}CD25^{+}$ T cells developed diabetes albeit with delayed kinetics (Fig. 5C). In contrast, NOD.*scid* mice receiving NOD.B6Idd3 $CD4^{+}CD25^{+}$ T cells plus diabetogenic splenocytes exhibited a significantly delayed onset and reduced frequency of diabetes relative to recipients of the cell mixture containing NOD $CD4^{+}CD25^{+}$ T cells (Fig. 5C). Therefore $CD4^{+}CD25^{+}$ T cells from NOD.B6Idd3 mice exhibit an increased suppressor activity compared to NOD $CD4^{+}CD25^{+}$ T cells.

To determine whether the protection mediated by NOD.B6Idd3 $CD4^{+}CD25^{+}$ T cells was due to quantitative or qualitative differences within the pool of $CD62L^{HI}FoxP3^{+}$Treg, the suppressor activity of these immunoregulatory effectors was tested *in vitro*. $CD62L^{LO}$- and $CD62L^{HI}$-expressing $CD4^{+}CD25^{+}$ T cells were FACS sorted from the PLN of 16 wkold NOD.B6Idd3 and NOD female mice, and then cultured at various ratios with naïve $CD4^{+}$ T cells from the spleen of NOD mice. As expected, $CD62L^{LO}CD4^{+}CD25^{+}$ T cells from either NOD.B6Idd3 or NOD female mice were inefficient at suppressing proliferation of the stimulated $CD4^{+}$ T cells (Fig. 5D). On the other hand, $CD62L^{HI}CD4^{+}CD25^{+}$ T cells effectively suppressed proliferation of the responder $CD4^{+}$ T cells. Furthermore, no significant difference in suppressor activity of NOD.B6Idd3 and NOD $CD62L^{HI}FoxP3^{+}$Treg was detected (Fig. 5D). Therefore, the enhanced suppressor activity detected in the PLN of NOD.B6Idd3 mice is due to an increased number of

CD62L^{HI}FoxP3^{+}Treg, consistent with results obtained in the above co-adaptive transfer experiments (Fig. 5C).

The frequency of CD62L^{HI}FoxP3^{+}Treg is increased *in vivo* in NOD mice treated with IL-2

Since IL-2 secretion by conventional T cells is limited in NOD mice compared to NOD.B6Idd3 animals (Supplementary Figure 1) [38], then increasing the level of "endogenous" IL-2 would be expected to enhance the frequency of CD62L^{HI}FoxP3^{+}Treg *in vivo*. To test this hypothesis 10 wk-old NOD female mice were injected intramuscularly with a doxycycline inducible AAV recombinant encoding IL-2 (AAVTet-IL-2). No difference was detected in the frequency of CD4^{+}CD25^{+}Foxp3^{+} T cells in AAV-Tet-IL-2 treated but uninduced NOD mice or animals left untreated (Fig. 6A,B). In contrast, NOD mice treated with AAV-Tet-IL-2 and in which IL-2 transgene expression was induced exhibited an increased frequency of CD4^{+}CD25^{+}Foxp3^{+} in all tissues tested (Fig. 6A,B), and showed a significant increase in CD62L^{HI}-expressing CD4^{+}CD25^{+}Foxp3^{+} T cells in the PLN (Fig. 6C). Furthermore, addition of IL-2 to FACS-sorted CD62L^{LO}-expressing CD4^{+}CD25^{+} T cells up-regulated expression of CD62L *in vitro* (Fig. 6D). These results indicate that: 1) IL-2 availability *in vivo* regulates the frequency of CD62L^{HI}FoxP3^{+}Treg, and 2) IL-2 can "convert" CD62L^{LO}FoxP3^{+}Treg into CD62L^{HI}FoxP3^{+}Treg *in vitro*.

DISCUSSION

Analyses of NOD mice congenic for protective *Idd3* intervals have shown that aberrant expression of IL-21 and IL-2 influences various aspects of β cell autoimmunity in NOD mice [34-38]. Increased expression of IL-21 and IL-21R by T cells is associated with enhanced development of pathogenic T effectors in NOD mice through for instance, disruption of T cell homeostasis [34, 36, 40-42]. IL-21 has also been reported to render conventional T cells resistant to the suppressor effects of FoxP3^{+}Treg [43, 44]. In NOD mice the resistance of conventional T cells to Treg-mediated suppression, however, appears to be independent of *Idd3* [45]. On the other hand, decreased transcription of the *Il2* gene in NOD mice has been linked to a reduced frequency of FoxP3^{+}Treg in the PLN, decreased intra-islet survival, a limited suppressor function of FoxP3^{+}Treg, in addition to an impaired capacity of FoxP3^{+}Treg to expand in the islets [24, 37, 38]. Differences in glycosylation of IL-2 between B6 and NOD mice, however, have no effect on diabetes development [46]. The current study provides new insight into how dysregulation of IL-2 adversely influences the pool of FoxP3^{+}Treg in NOD mice as T1D progresses. We show that reduced IL-2 expression in NOD mice is associated with a temporal shift favoring CD62L^{LO}- versus CD62L^{HI}-expressing FoxP3^{+}Treg (Fig. 3) thereby altering the composition and diminishing the suppressor function of the overall pool of FoxP3^{+}Treg (Fig. 5).

Previous work by our group [7] and others [38] demonstrated that the progression of β cell autoimmunity correlates with an age-dependent decrease in the frequency of CD62L^{HI}FoxP3^{+}Treg in NOD female mice. The current study shows that this decrease is due to an inverse relationship between CD62L^{HI}- and CD62L^{LO}-expressing FoxP3^{+}Treg that is dependent on the level of IL-2 expression. A direct role for IL-2 in regulating the balance between CD62L^{HI}FoxP3^{+}Treg and CD62L^{LO}FoxP3^{+}Treg was seen *in vitro* and *in vivo*. Supplementing cultures of sorted CD62L^{LO}CD4^{+}CD25^{+} T cells with IL-2 for instance, increased the frequency of CD62L^{HI}CD4^{+}CD25^{+} T cells (Fig. 6D). In addition, an increase in the frequency of CD62L^{HI}FoxP3^{+}Treg was detected in the PLN of NOD mice following a brief induction of AAV encoded IL-2 (Fig. 6C). This *in vivo* pulse of ectopic IL-2 also resulted in effective suppression of β cell autoimmunity and prevention of overt diabetes in treated NOD mice (K.S.G., M.C.J. & R.T.; unpublished results).

The above results are consistent with IL-2 providing critical signals for the maintenance of the FoxP3$^+$Treg compartment in general [24, 25], and specifically CD62L^{HI}FoxP3$^+$Treg. Our findings demonstrate that the temporal shift in the composition of FoxP3$^+$Treg in NOD mice correlates with the proliferative status of CD62L^{HI}- versus CD62L^{LO}- expressing FoxP3$^+$Treg. In the islets of NOD mice a >2-fold increase in the frequency of proliferating cells is detected in CD62L^{LO} (45%) versus CD62L^{HI} (17%) -expressing FoxP3$^+$Treg (Fig. 4A,B). However, the frequency of proliferating CD62L^{HI}FoxP3$^+$Treg is increased 2-fold in the islets of NOD.B6Idd3 (33%) versus NOD (17%) mice (Fig. 4A,B), resulting in a significantly increased ratio of dividing CD62L^{HI}FoxP3$^+$Treg to CD62L^{LO}FoxP3$^+$Treg in NOD.B6Idd3 islets (Fig. 4C). A similar trend was detected in the islets of NOD mice treated with AAV-Tet-IL-2 and fed doxycycline (Supplementary Fig. 2). Increased proliferation in NOD.B6Idd3 mice would be expected to promote more efficient maintenance of the CD62L^{HI}FoxP3$^+$Treg pool compared to NOD mice. Although the frequency of proliferating CD62L^{LO}FoxP3$^+$Treg was also increased in the islets of NOD.B6Idd3 (55%) versus NOD (45%) mice, the difference between the two was not as great as that seen between the respective CD62L^{HI}FoxP3$^+$Treg pools (Fig. 4A,B). This finding suggests that CD62L^{HI}FoxP3$^+$Treg are more sensitive to changes in the level of IL-2 than CD62L^{LO}FoxP3$^+$Treg. Elevated IL-2 expression by conventional T cells in NOD.B6Idd3 mice may therefore selectively increase proliferation (Fig. 4) and survival [24] of suppressor-efficient CD62L^{HI}FoxP3$^+$Treg residing in the islets. IL-2 also has direct effects on CD62L^{LO}FoxP3$^+$Treg. As noted above, IL-2 converts a significant number of sorted CD62L^{LO}FoxP3$^+$Treg into CD62L^{HI}FoxP3$^+$Treg *in vitro* (Fig. 6D), possibly reflecting down-regulation of the activation status of CD62L^{LO}FoxP3$^+$Treg. Indeed, IL-2 mediates both positive and negative effects on conventional T cells depending on the activational status of the cells [28, 47]. Finally, APC may also influence the CD62L^{HI}FoxP3$^+$Treg to CD62L^{LO}FoxP3$^+$Treg ratio *in vivo*. The type and activational status of professional APC can have a marked effect on FoxP3$^+$Treg induction/expansion. Groups have shown that macrophages and DCs exhibit an increased tolerogenic capacity in NOD.Idd3 versus NOD mice [48, 49]; the mechanistic basis for this enhanced tolerogenic effect, however, has yet to be determined.

Recent studies with NOD.Idd3 congenic lines have shown that NOD-derived FoxP3$^+$Treg exhibit an impaired suppressor function [37, 38]. Our results demonstrate that the limited suppressor activity reported for NOD FoxP3$^+$Treg is due to an increased number and frequency of suppressor-deficient CD62L^{LO}FoxP3$^+$Treg, which “dilute out” the suppressor-competent CD62L^{HI}FoxP3$^+$Treg. The limited suppressor function of sorted NOD or NOD.B6Idd3 CD62L^{LO}FoxP3$^+$Treg was demonstrated *in vitro* (Fig. 5D), consistent with an earlier report [7]. These results, however, differ from work published by Szanya *et al.* which demonstrated that CD62L^{HI}CD4$^+$CD25$^+$ and CD62L^{LO}CD4$^+$CD25$^+$ T cells from the spleen of NOD mice differ in suppressor activity only in *in vivo,* but not *in vitro* assays [19]. The level of anti-CD62L Ab-binding and the gating scheme may account for differences in the frequency of and in turn the *in vitro* suppressor activity of the pool of CD62L^{LO}FoxP3$^+$Treg in the respective studies. In addition, Szanya *et al.* examined splenic-derived CD62L^{LO}FoxP3$^+$Treg, whereas in this study CD62L^{LO}FoxP3$^+$Treg were prepared from PLN; “tissue residency” may also influence the suppressor activity of these T cells and contribute to the disparity between the studies. Reduced TGFβ1 [7] expression relative to CD62L^{HI}FoxP3$^+$Treg, however, is consistent with a diminished suppressor activity by CD62L^{LO}FoxP3$^+$Treg. In contrast to NOD mice, the increased frequency of CD62L^{HI}FoxP3$^+$Treg in the PLN and islets of NOD.B6Idd3 mice efficiently blocks β cell autoimmunity (Fig. 5B,C). Supporting this model is the marked increase in the ratio of FoxP3$^+$Treg to T effectors detected in the PLN and islets of NOD.B6Idd3 mice relative to age-matched NOD female mice (Fig. 5A). In addition, CD4$^+$CD25$^+$ T cells from the PLN of NOD.B6Idd3 mice proved to be more effective at suppressing the adoptive transfer of

diabetes relative to NOD $CD4^+CD25^+$ T cells (Fig. 5C). One caveat with the latter finding is that despite similar numbers of activated T effectors (e.g. $FoxP3^-CD4^+CD25^+$ T cells) in the transferred NOD and NOD.B6Idd3 $CD4^+CD25^+$ T cells, an increased frequency of β cell-specific pathogenic effector T cells may have limited the efficacy the NOD Treg pool. A previous study, however, showed that proliferation of transferred diabetogenic $CD4^+$ T cells was significantly reduced in the PLN of NOD.B6Idd3 versus NOD recipients [38], which is consistent with NOD.B6Idd3 mice having enhanced suppressor activity. Noteworthy is that no difference was detected in the *in vitro* suppressor activity of $CD62L^{HI}FoxP3^+$Treg from NOD and NOD.B6Idd3 mice (Fig. 4C); in addition similar *in vivo* suppressor activity was detected for the respective $CD62L^{HI}FoxP3^+$Treg as determined by co-adoptive transfer experiments (M.C.J. & R.T.; unpublished data). These observations argue that quantitative and not qualitative differences in $CD62L^{HI}FoxP3^+$Treg explain the distinct suppressor activity of the $FoxP3^+$Treg pool detected in NOD and NOD.B6Idd3 mice (Fig. 5B). It is important to note that the frequency of $CD62L^{HI}FoxP3^+$Treg decreased with age in the islets of NOD.B6Idd3 albeit to a lesser extent than seen in NOD islets (Fig. 3D). NOD.B6Idd3 mice develop insulitis and diabetes but at a reduced frequency and a delayed onset compared to NOD mice (Fig. 1). Therefore, in addition to IL-2 other factors contribute to the homeostasis and function of $CD62L^{HI}FoxP3^+$Treg.

In summary we demonstrate that reduced IL-2 expression impacts $FoxP3^+$Treg in NOD mice by altering the ratio of $CD62L^{HI}$ to $CD62L^{LO}$ $FoxP3^+$Treg and in turn reducing the suppressor activity of the $FoxP3^+$Treg compartment. These findings provide further rationale for the development of IL-2-based immunotherapy as a means to manipulate $FoxP3^+$Treg for the prevention and suppression of β cell autoimmunity.

MATERIALS & METHODS

Mice

NOD/LtJ and NOD.CB17-Prkdc^{scid}/J (NOD.*scid*) mice were maintained and bred under pathogen-free conditions in an American Association for Laboratory-accredited animal facility. NOD.B6c3D mice, provided by Dr. Ed Leiter (The Jackson Laboratory) were established by introgression of an ~17 Mb region of the *Idd3* interval derived from B6 mice (NOD.B6Idd3) for 13 backcross generations. The length of the congenic interval was determined by typing with MIT microsatellite markers and using the MGI posting data from NCBI Build 37 (Supplementary Table 1). Mice were monitored for diabetes by measuring urine glucose levels. All procedures were approved by the University of North Carolina Animal Use and Care Committee.

T cell isolation and culturing conditions

Single cell suspensions were prepared from the thymus, pancreatic lymph nodes (PLN), and spleen, and filtered with a 70 μM strainer (Fisher Scientific). PBL were obtained via submandibular puncture using lancets (Golden Rod) and RBC lysed with ACK solution. Islet infiltrating cells were isolated from purified, hand-picked islets. Briefly, pancreases were digested with 2.0 mg/ml Collagenase P (Roche) for 20 min at 37°C, and islets purified on a Ficoll (Sigma-Aldrich) gradient. Lymphocytes infiltrating the islets were harvested by dissociating the islets using enzyme-free cell dissociation solution (Sigma-Aldrich).

Naïve $CD4^+$ T cells were isolated from splenocytes using a bead-based naïve CD4 T cell kit (Miltenyi Biotec). Briefly, total lymphocytes were incubated with a biotin-labeled Ab cocktail that selectively enriches for $CD4^+$ T cells but depletes $CD4^+CD25^+$ cells. Enriched $CD4^+CD25^-$ T cells were then incubated with CD62L-conjugated micro-beads and isolated using a magnetic column.

For general T cell cultures, 2×10^5 cells were resuspended in complete RPMI 1640 medium (Gibco) containing 10% heat-inactivated FBS, 100 U/ml penicillin/streptomycin (Gibco), and 50 uM 2-ME (Sigma-Aldrich). T cells were stimulated in 96-well plates coated with varying concentrations of purified anti-CD3 Ab (2C11, eBioscience) and soluble, functional-grade anti-CD28 Ab at 2 ug/ml (37.51, eBioscience). In some experiments supernatants were collected, diluted 1:3 in 1% BSA in PBS, and IL-2 secretion measured 24 h post stimulation. An anti-IL-2 Ab set (eBioscience) was used at 2 ug/ml on a high-binding ELISA plate (Costar).

FACS

Total cells from the respective tissues were stained with a variety of fluorochromeconjugated monoclonal Ab (mAb) including: anti-CD3 (2C11), anti-CD4 (L3T4), anti-CD8 (Ly-2), anti-CD25 (PC61.5), anti-CD44 (IM7), anti-CD62L (MEL14), and anti-FoxP3 (FJK.16 kit) (eBioscience). Fc receptors were blocked with a 1/200 dilution of rat Ig prior to staining. Intracellular Ki67 (B56; BD Biosciences) staining was done using cytofix/cytoperm reagents (BD Biosciences) according to manufacturer's specifications. Data were acquired on a Cyan flow cytometer (DakoCytomation), and analyzed using Summit software (DakoCytomation). In addition $CD4^+CD25^+$ T cells ($CD62L^{LO}$ or $CD62L^{HI}$) were sorted by a MoFlo high-speed sorter (DakoCytomation).

Intracellular cytokine staining was preformed on single cell suspensions from PLN or islet-infiltrating cells as previously described [50]. Briefly, lymphocytes were stimulated with 10 ng/ml PMA (Sigma-Aldrich) and 150 ng/ml ionomycin (Sigma-Aldrich) in complete RPMI 1640 medium for 6 h at 37°C; 10 ug/ml of Brefeldin A (Sigma-Aldrich) was added for the final 4 h of incubation. Cells were stained for surface molecules, fixed and permeabilized with cytokfix/cytoperm reagents (BD Biosciences), and stained for intracellular IFN-γ (XMG1.2) (eBioscience).

In vitro suppression assay

Different ratios of FACS-sorted $CD4^+CD25^+CD62L^{LO/HI}$ T cells were cultured with cell-trace violet (Invitrogen) labeled naïve 5×10^4 $CD4^+$ T cells, 2 ug/ml anti-CD28, and 2 μg/ml anti-CD3 Ab in 96-well round bottom plates (Costar) for 3 d. Proliferation was assessed in triplicate by FACS analysis as the total percentage of labeled $CD4^+Thy1.2^+$ naïve cells undergoing at least one round of division.

Cell adoptive transfers

Diabetogenic NOD splenocytes (2.5×10^6) were suspended in PBS and injected i.p. into 8 wk-old NOD.*scid* male mice alone or in combination with FACS sorted $CD4^+CD25^+$ T cells (1×10^5) isolated from the PLN of NOD or NOD.B6Idd3 mice. Mice were monitored bi-weekly post-transfer for diabetes.

Construction of and treatment with adeno-associated virus encoding NOD IL-2

Using the forward primer 5′-gaagcttcaggcatgtacagcatgcagctc-3′ that includes a HindIII restriction site and the reverse primer 5′-gtcgactagttattgagggcttgttgagat-3′ that contains an EcoRV restriction site, the *Il2* gene was PCR amplified with PFU Turbo (Promega) from mRNA (Qiagen) of ConA (Sigma-Aldrich) stimulated NOD lymphocytes. Amplicons were subcloned into the topo-TA vector (Invitrogen) and sequenced. Full-length cDNA encoding *Il2* was subcloned into an adeno-associated virus (AAV)-Tet-on vector plasmid (kindly provided by Dr. Sihong Song) using SalI and EcoRV sites. Transgene expression was verified by measuring via ELISA IL-2 secretion by HEK 293 cells transfected with AAV-Tet-on-IL-2 plasmid DNA.

AAV virus production was previously described [51]. Briefly, packaged AAV serotype 1 (AAV1) virus was prepared by transfecting 293 cells via calcium phosphate with the adeno helper encoding plasmid (pXX6-80), AAV1 encoding plasmid (pXR-1), and the Tet-on-IL-2 constructs (described above). Nuclear fractions were harvested and virus purified with an iodixonal (Sigma-Aldrich) gradient. The virus containing fractions and titer were determined by Southern dot blot.

NOD female mice were vaccinated with 5×10^{10} viral particles of AAV-Tet-on-IL-2 virus serotype 1 (AAV-Tet-IL-2) in contra-lateral, hind limb muscles using an insulin syringe. After injection, mice were fed chow containing 200 mg/kg doxycycline (BioServ) for 2 wks.

Insulitis scoring

Pancreases were harvested and fixed with formalin for 24 h. Serial sections 90 um apart were prepared and stained with H&E. More than 100 islets were scored per group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AAV	adeno-associated virus
GITR	glucocorticoid-induced TNF receptor
Treg	immunoregulatory T cells
Idd	insulin-dependent diabetes
PLN	pancreatic lymph node
T1D	Type 1 diabetes

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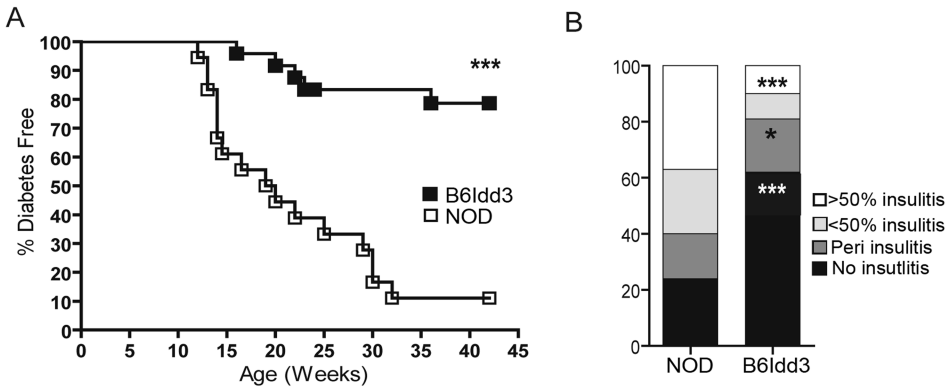

Figure 1. NOD.B6Idd3 female mice exhibit a reduced frequency of diabetes and insulitis compared to NOD female mice

(A) Female NOD (open square, n=20) and NOD.B6Idd3 (black square, n=19) were monitored for diabetes incidence. ***, $p<0.001$ (Kaplan-Meier Log Rank Test). (B) The frequency of insulitis in the pancreas of 16 wk old NOD (n=7) and NOD.B6Idd3 (n=6) female mice was determined via H&E staining. * $p<0.05$, *** $p<0.001$; NOD versus NOD.B6Idd3 for a given type of insulitis (Chi Square).

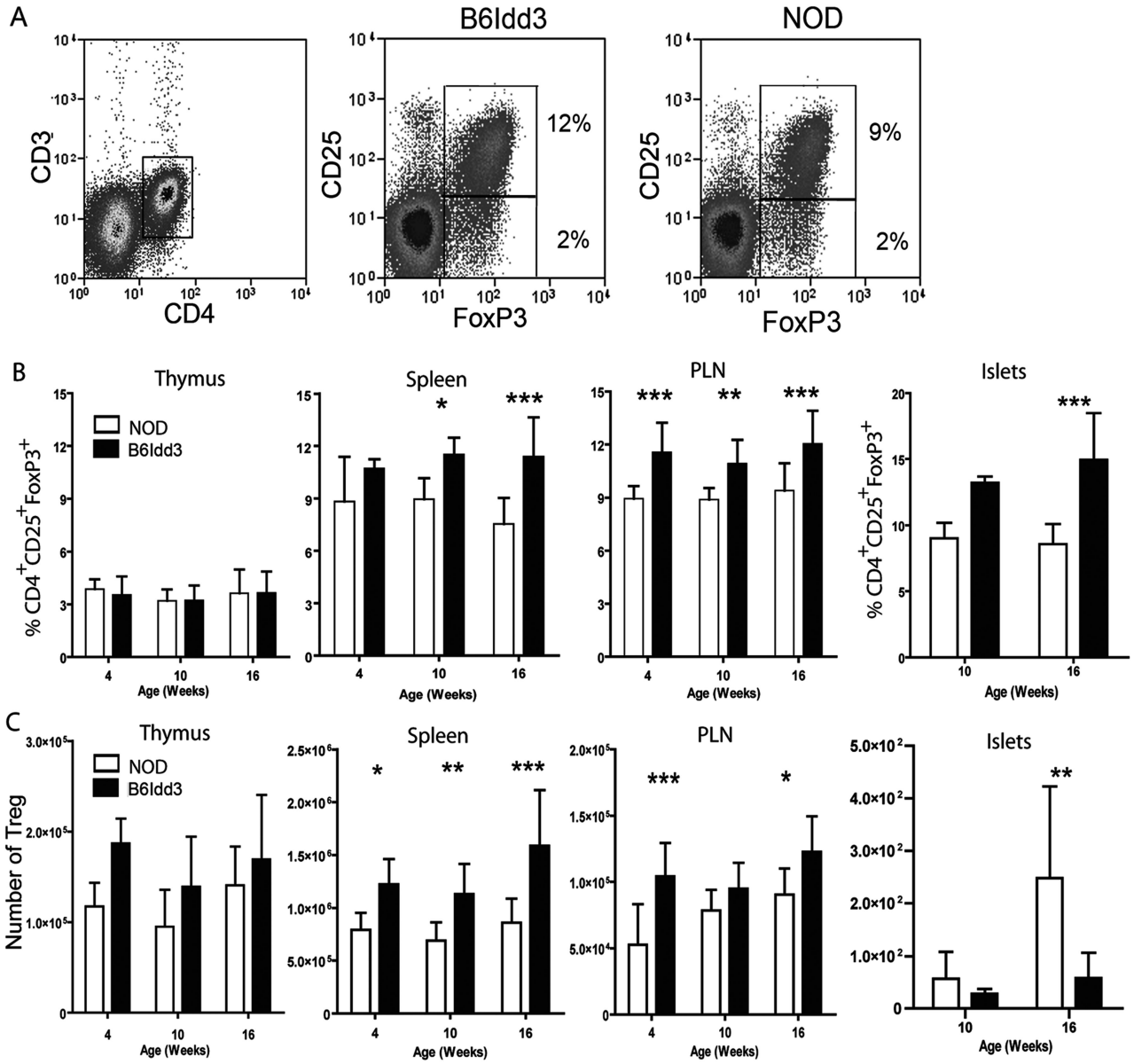

Figure 2. NOD.B6Idd3 mice have an increased frequency of peripheral FoxP3+Treg compared to age-matched NOD mice

(A) Representative FACS plots of NOD and NOD.B6Idd3 PLN at 16 wks of age staining for CD3+CD4+ T cells (left column) used to gate CD25+FoxP3+ T cells (right columns) with the average percentage of the indicated populations shown to the right of each gate. The (B) frequency and (C) absolute number of CD3+CD4+CD25+FoxP3+ T cells in the thymus, spleen, PLN and islets were measured in female NOD (n=8-15/age group) and NOD.B6Idd3 mice (n=7-16/age group) at 4, 10 and 16 wks of age. The absolute number in each respective tissue was determined by multiplying the total number of counted cells by the percentage of T cells determined by FACS. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; NOD.B6Idd3 versus NOD for a given tissue (2-way ANOVA, data are mean ±SEM).

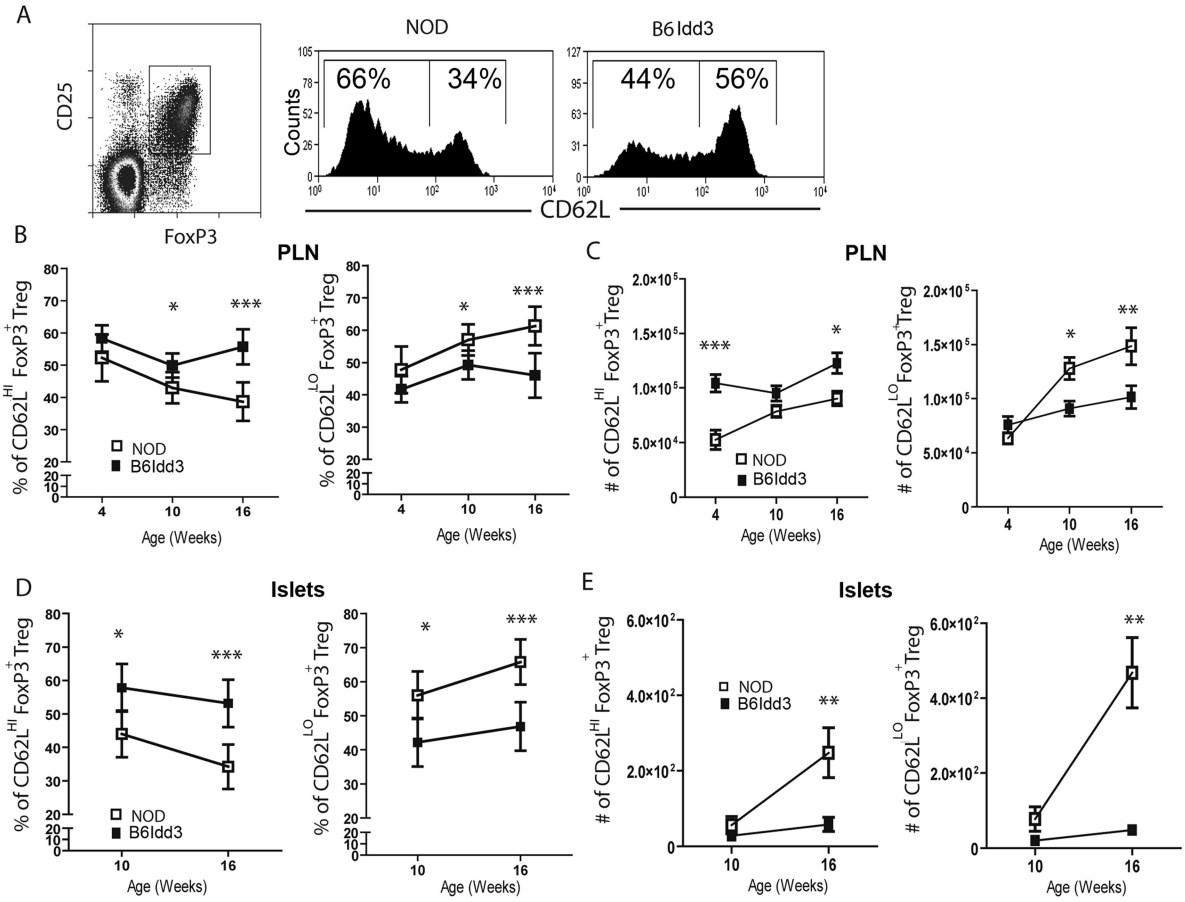

Figure 3. A temporal shift in CD62L$_{HI}$- versus CD62L$_{LO}$-expressing FoxP3+Treg is detected in NOD but not NOD.B6Idd3 female mice

(A) Representative FACS staining profile of CD62L expression on gated CD3+CD4+CD25+FoxP3+ T cells in the PLN of 16 wk-old NOD and NOD.B6Idd3 mice. The (B, D) frequency and (C, E) number of CD62L$_{HI}$- and CD62L$_{LO}$-expressing CD3+CD4+CD25+FoxP3+ T cells in the (B, C) PLN and (B, C) islets of 4, 10 and/or 16 wk-old NOD (n=7-10/age group) and NOD.B6Idd3 (n=8-10/age group) female mice. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; NOD.B6Idd3 versus NOD for a given tissue (2-way ANOVA, data are ±SEM).

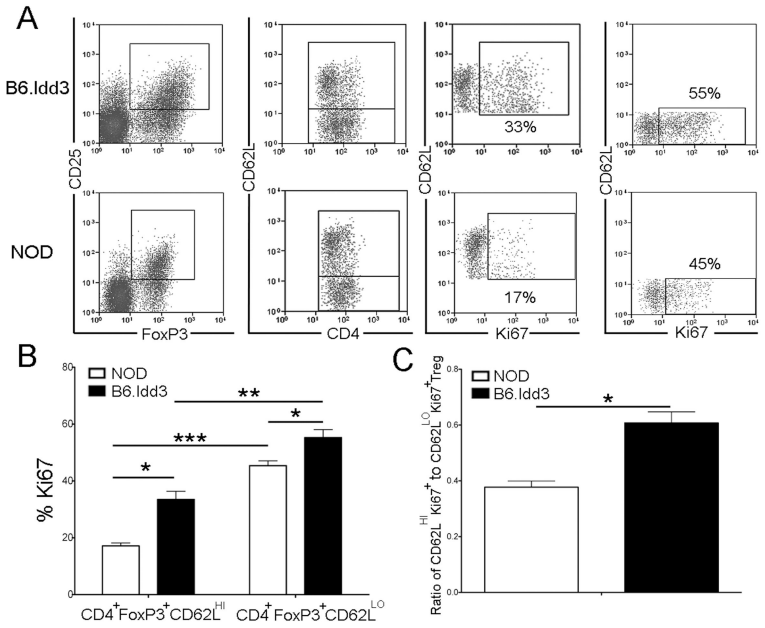

Figure 4. Proliferation of CD62L^{HI}FoxP3+Treg is increased in the islets of NOD.B6Idd3 versus NOD female mice

(A) Representative FACS plots for the gating scheme used to assess the frequency of islet infiltrating Ki67-staining CD62L$_{HI}$ versus CD62L$_{LO}$ in CD4+CD25+FoxP3+ T cells of 12 wk-old NOD and NOD.B6Idd3 female mice. (B) Average percent and (B) the ratio of Ki67-staining CD62L$_{HI}$CD4+CD25+FoxP3+ and CD62L$_{LO}$CD4+CD25+FoxP3+ T cells from the islets of 12 wk-old NOD (n=5) and NOD.B6Idd3 (n=5) female mice. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (2-way ANOVA, data are + SEM).

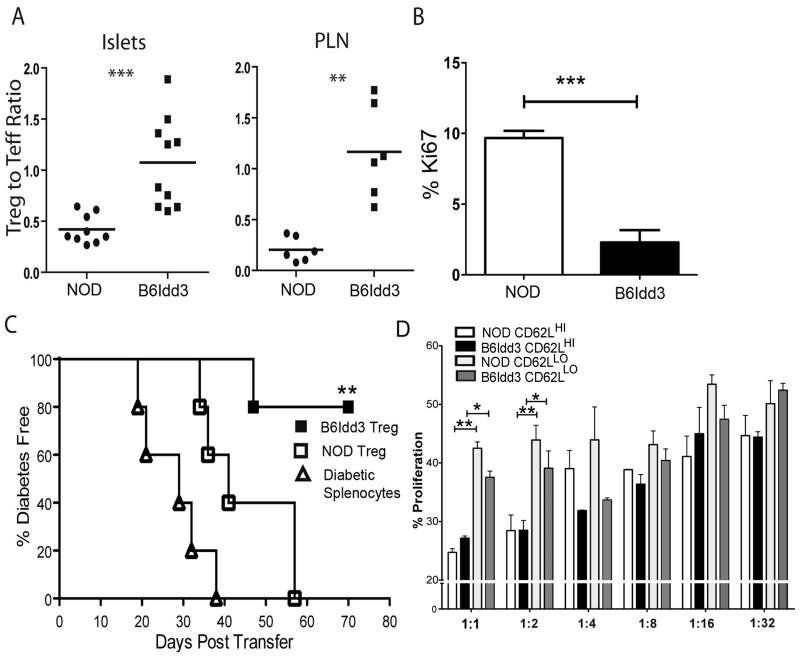

Figure 5. The FoxP3+Treg pool in 16 wk-old NOD.B6Idd3 versus NOD female mice exhibits increased suppressor activity

(A) The ratio of CD62L$_{HI}$-expressing CD3+CD4+CD25+FoxP3+ T cells to CD3+CD4+ T cells expressing intracellular IFNγ (e.g. type 1 T effectors (Teff)) was determined in the islets and PLN of 16 wk-old NOD (n=9 and n=6, respectively) and NOD.B6Idd3 (n=10 and n=6, respectively) female mice via FACS. Each data point represents an individual mouse, horizontal bar represents the mean. **, $p<0.01$; ***, $p<0.001$; NOD.B6Idd3 versus NOD (Student's t Test). (B) Ki67 staining for T cell proliferation of CD3+ T cells in the islets of 16 wk-old NOD.B6Idd3 (n=6) versus NOD (n=5) female mice; ***, $p<0.001$ (Students t Test). (C) CD4+CD25+ T cells sorted from pooled PLN of 16 wk-old NOD or NOD.B6Idd3 female mice were transferred with diabetogenic NOD splenocytes into NOD.*scid* mice (n=5 per group) and diabetes monitored; as a control diabetogenic splenocytes alone were transferred into NOD.*scid* recipients; **, $p<0.001$, NOD.B6Idd3 versus NOD CD4+CD25+ T cells (Kaplan-Meier Log Rank Test). (D) The *in vitro* suppressor activity was compared between CD62L$_{HI}$- and CD62L$_{LO}$-expressing CD4+CD25+ T cells sorted from the PLN of 16 wk-old NOD and NOD.B6Idd3 female mice. Suppressor activity was determined by measuring via FACS proliferation of celltrace violet dye labeled CD4+CD25− responder T cells stimulated with anti-CD3 plus - CD28 Ab, and cultured with varying numbers of CD62L$_{HI}$- or CD62L$_{LO}$-expressing CD4+CD25+ T cells; data are the average percent of proliferation from 2 wells±SEM. **, $p<0.01$; *, $p<0.05$; CD62L$_{HI}$ versus CD62L$_{LO}$ in NOD and NOD.B6Idd3 (2-way ANOVA,). Data are representative of three independent experiments.

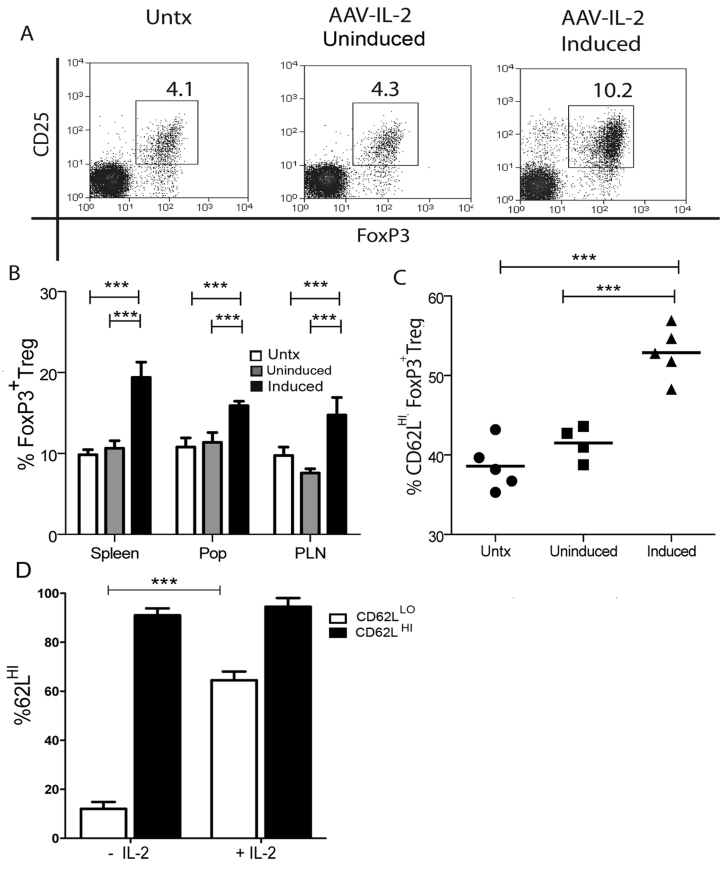

Figure 6. Increased IL-2 induces an elevated frequency of CD62L$_{HI}$FoxP3+Treg *in vivo* and *in vitro*

(A) A representative FACS plot of the frequency of CD3+CD4+ gated T cells expressing CD25 and FoxP3 in PBL prepared from NOD female mice left untreated (Untx; n=5) or injected at 10 wks of age with AAV-Tet-IL-2 and given doxycycline containing chow for 2 wk (induced; n=5) or not (uninduced; n=4) 3 wks-post induction (average percentages per group are inlayed in the dot plots). Similarly the frequency of (B) CD3+CD4+CD25+FoxP3+ T cells in the spleen, popliteal lymph nodes (Pop) and PLN, and (C) CD62L$_{HI}$- expressing CD3+CD4+CD25+FoxP3+ T cells in the PLN were determined via flow cytometry in the respective groups of NOD mice. (C) Each data point represents an individual mouse, horizontal bars represent the mean. ***, $p<0.001$ (2-way ANOVA, data are±SEM). (D) CD62L expression after culturing for 48 h sorted CD62L$_{HI}$ or CD62L$_{LO}$ CD3+CD4+CD25+ T cells in the presence or absence of 20 ng/ml of IL-2 . ***, $p<0.001$, CD62L$_{LO}$ (−IL-2) versus CD62L$_{LO}$ (+IL-2) (Student's t Test, data are ±SEM).