Published in final edited form as:

Sci Immunol. 2017 March 31; 2(9): . doi:10.1126/sciimmunol.aah4569.

LAG-3 limits regulatory T cell proliferation and function in autoimmune diabetes

Qianxia Zhang^{1,2}, Maria Chikina³, Andrea L. Szymczak-Workman^{1,2}, William Horne⁴, Jay K. Kolls⁴, Kate M. Vignali^{1,2}, Daniel Normolle⁵, Maria Bettini^{2,†}, Creg J. Workman^{1,2}, and Dario A.A. Vignali^{1,2,6,*}

¹Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

²Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN 38105

³Department of Computational and Systems Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

⁴Richard King Mellon Foundation Institute for Pediatric Research, Children's Hospital of Pittsburgh of University of Pittsburgh Medical Center, Pittsburgh, PA 15224

⁵Department of Biostatistics, University of Pittsburgh, Pittsburgh, PA 15232

⁶Tumor Microenvironment Center, University of Pittsburgh School of Medicine, Pittsburgh, PA 15232

Abstract

Inhibitory receptors are pivotal in controlling T cell homeostasis because of their intrinsic regulation of conventional effector T (Tconv) cell proliferation, viability and function. However, the role of Inhibitory receptors on regulatory T (Treg) cells remains obscure, as they could be required for suppressive activity and/or limit Treg cell function. We evaluated the role of

Corresponding author. dvignali@pitt.edu.

Current address: Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030

Author contributions

Q.Z. designed and performed most of the experiments, carried out statistical analyses and wrote the manuscript. M.C. performed computational analysis of the RNAseq data. A.L.S. designed and made the *Lag3*^{ΔL-YFP} construct and generated the *Lag3*^{ΔL-YFP} mice. W.H. and J.K.K. sequenced RNA-cDNA libraries and performed initial data normalization. K.M.D assisted in designing targeting constructs. D.N. oversaw the statistical analyses. M.B. provided input in experiment design and data analysis. C.J.W. assisted in designing the mouse construct, experimental design and analysis. D.A.A.V. conceived the project, directed the research and wrote the manuscript. All authors edited and approved the manuscript.

Competing interests

D.A.A.V. and C.J.W. are inventors on issued patents (US [8,551,481]; Europe [1897548]; Australia [200427526]; Hong Kong [1114339]) held by St Jude Children's Research Hospital and Johns Hopkins University that cover LAG3. Additional application pending in Japan.

Data and materials availability

The RNAseq data have been deposited in the Gene Expression Omnibus at the National Center for Biotechnology Information with accession number GSE94581. The *Lag3*^{ΔL-YFP} NOD mice will be freely distributed to investigators at academic institutions for non-commercial research when an MTA is signed. These mice will also be distributed to commercial entities upon completion of a licensing agreement with the University of Pittsburgh and St. Jude Children's Research Hospital. Individual requests for shipment of mice to AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) accredited institutions will be honored. The recipient investigators should provide written assurance and evidence that the animals will be used solely in accord with their local IACAC review, that animals will not be further distributed by the recipient without consent from the University of Pittsburgh, Office of Research, and that animals will not be used for commercial purposes.

Lymphocyte Activation Gene-3 (LAG3, CD223) on Treg cells by generating mice in which LAG3 is absent on the cell surface of Treg cells in a murine model of Type 1 Diabetes. Surprisingly, mice that lacked LAG3 expression on Treg cells exhibited reduced autoimmune diabetes, consistent with enhanced Treg cell proliferation and function. Whereas the transcriptional landscape of peripheral wild-type (WT) and *Lag3*-deficient Treg cells was largely comparable, substantial differences between intra-islet Treg cells were evident and involved a subset of genes and pathways that promote Treg cell maintenance and function. Consistent with these observations, *Lag3*-deficient Treg cells out-competed WT Treg cells in the islets but not in the periphery in co-transfer experiments due to enhanced IL2-Stat5 signaling and increased Eos expression. Our study suggests that LAG3 intrinsically limits Treg cell proliferation and function at inflammatory sites, promotes autoimmunity in a chronic autoimmune-prone environment and may contribute to Treg cell insufficiency in autoimmune disease.

INTRODUCTION

Inhibitory receptors (IRs) are pivotal in controlling and shaping the host immune response. Insufficient co-inhibition can lead to the breakdown of self-tolerance, whereas chronic utilization of inhibitory pathways constitutes a major barrier to effective anti-tumor immunity (1–3). Tumor-infiltrating lymphocytes (TILs) up-regulate multiple inhibitory receptors and exhibit an exhausted T cell phenotype as a consequence of chronic antigen stimulation (3). Recent clinical trials have highlighted the therapeutic benefit of IR blockade, so called checkpoint inhibition (eg. anti-CTLA4, anti-PD1, anti-PD-L1) (2, 3). However, not all patients benefit leading to speculation that compensatory mechanisms may be elevated in non-responders and that additional inhibitory receptors may need to be blocked. Lymphocyte Activation Gene-3 (LAG3) is the most recent IR to be targeted in the clinic.

LAG3 is expressed on activated T cells, intrinsically limiting conventional T (Tconv) cell proliferation, expansion and viability (4–7). LAG3 is required for maintaining self-tolerance in an autoimmune-prone environment (1, 8). Mice deficient in *Lag3* on the Non-Obese Diabetic (NOD) background exhibit accelerated autoimmune diabetes with 100% penetrance (8). In contrast, LAG3 contributes to tumor-mediated immune suppression and promotes tumoral immune escape (9, 10). Whereas LAG3 blockade or deficiency alone only has a minimal effect on tumor growth in mouse models, combinatorial PD1 blockade or deletion leads to tumor clearance (9).

LAG3, as well as other inhibitory receptors, are also highly expressed on regulatory T (Treg) cells (11–14), a critical suppressive sub-population of T cells that prevents autoimmunity but limits anti-tumor immunity (15, 16). This complicates analysis of studies using *Lag3*^{-/-} mice or LAG3 blocking antibodies and limits the dissection of IR function on different T cell populations. Previous studies have suggested that LAG3 was utilized as a mechanism for Treg cell suppression (11–13, 15). However, it is also possible that LAG3 may intrinsically limit Treg cell function in a manner commensurate to its role on other T cell populations. This ambiguity complicates defining the role of LAG3, and perhaps other inhibitory receptors, on Treg cells, assessing whether differential IR expression on Tregs

impacts the progression of autoimmune disease, and interpreting the mechanistic basis of LAG3 blockade in mouse models and patients with cancer.

As the activity of Treg cells is likely to be near maximal in tumors (17), the full impact of LAG3 deficiency on Treg cell function and viability may not be evident in tumor models. Thus, we chose to assess Treg cell function in a model in which their activity delays disease onset but is ultimately insufficient (18). Indeed, several studies have shown that Treg cells ultimately lose stability and function in autoimmune-prone environment (19–22). To assess the function of LAG3 on Treg cells, we generated mice that specifically lack LAG3 on Foxp3⁺ Treg cells and assessed their proliferation and function in a mouse model of autoimmune diabetes. These mice exhibited reduced autoimmune diabetes due to enhanced Treg cell proliferation and function in islets. The expression of LAG3 on Treg cells may limit Treg proliferation and function by down-regulating Eos and the IL2-Stat5 pathway by reduction of CD25 expression.

RESULTS

Intra-islet Treg cells constitutively express LAG3

To assess the role of LAG3 on Treg cells, we utilized the NOD mouse model of Type 1 Diabetes (T1D) (23). We first assessed the expression of LAG3, as well as other inhibitory receptors, on islet-infiltrating T cells using transcriptional analysis and flow cytometry. Multiple inhibitory receptors were transcriptionally up-regulated in intra-islet compared with peripheral Treg cells (Fig. 1A). Interestingly, LAG3 was substantially up-regulated on islet-infiltrating Treg cells compared to peripheral Treg cells in NOD mice, and the percentage of LAG3⁺ Treg cells was also higher than the percentage of LAG3⁺ CD4⁺ and CD8⁺ Tconv cells (Fig. 1, B to D, and fig. S1), suggesting a role of LAG3 on Treg cells.

The absence of LAG3 on Treg cells results in reduced autoimmune diabetes

To assess the importance of LAG3 expression on Treg cells in controlling autoimmune diabetes, we generated *Lag3*^{ΔL-YFP} conditional knockout-reporter mice (backcrossed to NOD/ShiLtJ for 12 generations – see Materials and Methods) that lack cell surface expression of LAG3, and thus cannot mediate signaling but continue to release soluble LAG3 (24, 25), specifically on Treg cells when crossed with the Foxp3^{Cre-GFP}.NOD mice (Fig. 2A, and fig. S2). Although there is no evidence that soluble LAG3 impacts T cell function (24, 25), we took this approach to avoid this complication. This mutant mouse also incorporated an IRES-YFP cassette inserted into the 3' UTR as a reporter of *Lag3* promoter activity (Fig. 2A). We initially crossed *Lag3*^{ΔL-YFP}.NOD with Cd4^{Cre}.NOD to assess the phenotype following global loss of surface LAG3 on all T cells (Fig. 2B, and fig. S2, B and C). Loss of LAG3 surface expression on all CD4⁺ and CD8⁺ T cells (*Lag3*^{ΔL-YFP}Cd4^{Cre}.NOD) resulted in dramatically accelerated onset of autoimmune diabetes with 100% penetrance by 12 week-of-age, which phenocopied our observations with *Lag3*^{-/-}.NOD mice and suggested that the dominant function of LAG3 was restricted to T cell populations (Fig. 2B) (8).

As LAG3 has been shown to be required for optimal Treg cell function (11–13, 15), we reasoned that the accelerated autoimmune diabetes observed in the *Lag3*^{-/-}.NOD and *Lag3*^{Δ/L-YFP}Cd4^{Cre}.NOD mice might be partially due to the loss of LAG3 expression on Treg cells. We then assessed the impact of the loss of LAG3 surface expression on Treg cells by analyzing *Lag3*^{Δ/L-YFP}Foxp3^{Cre-GFP}.NOD mice (fig. S2). Surprisingly, female *Lag3*^{Δ/L-YFP}Foxp3^{Cre-GFP}.NOD mice had significantly delayed onset of autoimmune diabetes, decreased diabetes incidence (48% vs. 84%) by 30 weeks-of-age, and male *Lag3*^{Δ/L-YFP}Foxp3^{Cre-GFP}.NOD mice were completely protected from autoimmune diabetes (Fig. 2C). Although we only assessed a small number of co-housed female *Lag3*^{+/-}L-YFPFoxp3^{Cre-GFP}.NOD littermates, their diabetes incidence was 60% suggesting that LAG3 exhibits haploinsufficiency. While the absence of LAG3 on Treg cells did not impact the degree of insulinitis at 6 weeks-of-age, it did lead to a significant reduction at 10 weeks-of-age in female and male *Lag3*^{Δ/L-YFP}Foxp3^{Cre-GFP}.NOD mice (Fig. 2D), suggesting that the expression of LAG3 on Treg cells had little impact on the initiation of islet infiltration but was critical to limit Treg-mediated self-tolerance and the onset of autoimmune diabetes. Taken together, these data suggest that LAG3 may limit Treg-mediated suppression of autoimmunity.

Consistent with the reduced insulinitis and diabetes observed, there were reduced numbers of CD4⁺Foxp3⁻ and CD8⁺ T cells in the islets of *Lag3*^{Δ/L-YFP}Foxp3^{Cre-GFP}.NOD mice (fig. S3A). While the number of Treg cells in the islets of *Lag3*^{Δ/L-YFP}Foxp3^{Cre-GFP}.NOD mice was decreased due to the reduced insulinitis and inflammation, there was a trend, albeit not reaching significance, towards a lower ratio of Tconv cells to Treg cells (fig. S3A). The proportion of Chromogranin A-specific (BDC2.5mi⁺) CD4⁺ T cells and islet specific glucose-6-phosphatase (IGRP)-specific (NRP-v7mi⁺) CD8⁺ T cells was not altered in the islets of *Lag3*^{Δ/L-YFP}Foxp3^{Cre-GFP}.NOD mice compared with controls, suggesting that *Lag3*-deficient Treg cells may not selectively suppress specific sub-populations of diabetogenic T cells but rather globally impact all islet-infiltrating cells (fig. S3B). This observation may have been anticipated given that we have previously shown that only islet antigen reactive T cells can enter the islets (26). The reduced number of Tconv cells in the islets of *Lag3*^{Δ/L-YFP}Foxp3^{Cre-GFP}.NOD mice was due to decreased CD8⁺ T cell proliferation [assessed by Ki67 expression and BrdU incorporation] and reduced expression of anti-apoptotic factor Bcl2 (27) in CD4⁺Foxp3⁻ T cells in islets (fig. S4 and S5). Both CD4⁺Foxp3⁻ and CD8⁺ T cells in the islets of *Lag3*^{Δ/L-YFP}Foxp3^{Cre-GFP}.NOD mice had significantly reduced expression of TNFα but not IFNγ (fig. S6). A significant reduction in IL2 production was also observed in CD4⁺Foxp3⁻ T cells in the islets of *Lag3*^{Δ/L-YFP}Foxp3^{Cre-GFP}.NOD mice (fig. S6). There was a trend, albeit not reaching significance, towards increased percentage of Th2 and Th17 cells (fig. S6). Although the activation, terminal differentiation [as marked by KLRG1 expression (28)], and proliferation of *Lag3*-deficient and WT Treg cells were comparable, *Lag3*-deficient Treg cells expressed higher level of the anti-apoptotic factor Bcl2 in the islets (fig. S5, and S7A). ICOS, which has been shown to be critical for Treg cell homeostasis and functional stability in NOD mice (29), was also up-regulated on intra-islet Treg cells in the absence of LAG3 (fig. S7A). Interestingly, the expression of multiple inhibitory receptors (PD1, TIGIT, and TIM3) was slightly enhanced on *Lag3*-deficient Treg cells (fig. S7A), inferring a cell intrinsic process in

Treg cells to compensate for the loss of LAG3. However, the suppressive capacity of intra-islet and peripheral WT and *Lag3*-deficient Treg cells in an *in vitro* micro-suppression assay was comparable on a per cell level (fig. S7B). Overall, these data suggest that *Lag3*-deficient Treg cells appear to have an enhanced impact on islet antigen-reactive T cell proliferation, effector cytokine production, and maybe viability (as suggested by Bcl2 expression) *in vivo*, perhaps at the population level due to their enhanced proliferative and survival capacity.

LAG3 impacts the Treg cell transcriptome

To assess the impact of LAG3 deletion on the Treg cell transcriptome, we performed RNA sequencing of WT and *Lag3*-deficient Treg cells from the islets and non-draining lymph nodes (ndLN). The whole genome expression profiles, as well as the expression of previously defined Treg cell signature genes (30, 31), were affected by the islet microenvironment (Fig. 1A and fig. S8). A substantial number of genes and pathways were modulated by the loss of LAG3 expression of Treg cells in the islets (Fig. 3A, fig. S9, and table S1). Interestingly, a group of genes were down-regulated in intra-islet WT but not *Lag3*-deficient Treg cells, compared to peripheral Treg cells (Fig. 3A, and fig. S10), suggesting that these genes might be required for optimal Treg cell function or survival, and that LAG3 was limiting their expression in intra-islet Treg cells.

One of these genes was *Ikzf4* (Eos), a co-repressor of Foxp3 that prevents the expression of Tconv genes in Treg cells (Fig. 3A, and fig. S10) (31–34). Strikingly, the expression profile of intra-islet WT Treg cells resembled the previously published transcriptional signature in *Ikzf4*-knockdown Treg cells, while the expression profile of intra-islet *Lag3*-deficient Treg cells resembled the transcriptional signature in mock control Treg cells (Fig. 3, A and B) (32). These data suggest that LAG3 might limit Eos expression, and thus the function and maintenance of intra-islet Treg cells. IL2 has been shown to be essential for Treg cell maintenance, whereas defective IL2 signaling in Treg cells triggers autoimmune islet destruction (22, 35, 36). Interestingly, genes modulated by IL2-STAT5 signaling were substantively enhanced in the absence of LAG3 on Treg cells (Fig. 3, C and D, and table S1). Overall, the transcriptome analyses suggest that LAG3 negatively regulates intra-islet Treg cells by down-regulating key genes and pathways that are essential for Treg maintenance and function.

LAG3 intrinsically limits Treg cell proliferation

To directly assess whether *Lag3*-deficient Treg cells had a proliferative advantage over WT Treg cells, and to determine if the pathways identified by transcriptome analysis were intrinsically regulated by LAG3 in Treg cells, we co-transferred an equal number of activated congenic marker-mismatched WT (Thy1.1⁺) and *Lag3*-deficient (Thy1.2⁺) Treg cells into NOD (Thy1.1⁺Thy1.2⁺) hosts (Fig. 4A). Both WT and *Lag3*-deficient donor Treg cells were sorted from mice that expressed the islet antigen-specific BDC2.5 TCR, which facilitated islet entry (26, 37). Foxp3 expression was unaltered in either Treg population following adoptive transfer (fig. S11, A and B), suggesting LAG3 may not impact Treg stability. Strikingly, *Lag3*-deficient Treg cells out-competed WT Treg cells in the islets (60% vs. 40%, respectively) and in the PLN (54% vs. 46%, respectively) but not in the periphery (Fig. 4, B and C).

Previous studies have shown that reduced CD25 and Bcl2 levels cause a decline in intra-islet Treg viability, while administration of low dose IL2 promotes Bcl2 expression and Treg survival (22, 38, 39). A higher percentage of intra-islet *Lag3*-deficient versus WT Treg cells expressed Ki67 and Bcl2 (Fig. 4, D and E, and fig. S11C). Although differences were also observed in periphery in these co-transfer experiments, this is probably due to the activation of Treg cells *in vitro* prior to adoptive transfer. Consistent with the transcriptomic analysis, *Lag3*-deficient Treg cells exhibited higher CD25 expression and STAT5 phosphorylation compared with WT Treg cells (Fig. 4, F and G, and fig. S11C). Furthermore, Eos (*Ikzf4*) expression was reduced in WT but not *Lag3*-deficient intra-islet Treg cells compared with periphery Treg cells, whereas another Ikaros family member Helios (*Ikzf2*) was unaffected by LAG3 expression (Fig. 4, H and I, and fig. S11C).

Finally, to determine if LAG3 modulated Eos expression and if direct modulation of Eos levels impacted Treg cell proliferation, we first assessed Eos levels in WT and *Lag3*-deficient Treg cells following stimulation *in vitro*. As anticipated from our transcriptomic analysis, *Lag3*-deficient Treg cells expressed more Eos following stimulation than WT Treg cells (Fig. 5). Likewise, activated *Lag3*-deficient Treg cells exhibited increased proliferation, as measured by BrdU incorporation, over WT Treg cells. Importantly, knockdown of *Ikzf4* in *Lag3*-deficient Treg cells reduced Eos and their proliferative capacity, whereas overexpression of *IKZF4* in WT Treg cells enhanced their proliferation (Fig. 5, and fig. S12). Taken together, these data support a model in which LAG3 intrinsically limits Treg cell proliferation and viability by modulating pathways that are critical for Treg cell function and proliferation, in particular the IL2/STAT5 and Eos pathways.

DISCUSSION

Our study supports a model in which the inhibitory receptor LAG3 intrinsically limits Treg proliferation and functionality by repressing pathways that promote the maintenance of Treg cells at inflammatory sites. *Lag3*-deficient Treg cells do not appear to have increased suppressive capacity on a per cell basis. However, they do have an enhanced proliferative and survival advantage that potentiates their suppressive capacity at the population level and endows them with a critical advantage over time. As disease progresses, subtle changes in *Lag3*-deficient Treg cells allow them to accumulate over time leading to a substantial impact on the development of chronic autoimmune diabetes. It is remarkable that this small, Treg-restricted genetic alteration renders male NOD mice resistant to diabetes and substantially limits autoimmune diabetes in female mice, while LAG3 deletion in all T cell subsets dramatically accelerates disease. Thus in autoimmune environments where chronic inflammation dominates, LAG3 may be constitutively expressed on Treg cells thereby limiting their capacity to block the function of diabetogenic T cells and prevent autoimmune diabetes. This raises the possibility that chronic IR expression on Treg cells may underlie their insufficiency in autoimmune disease. It is also possible that increased or chronic LAG3 expression on Treg cells may also limit their suppressive capacity in inflammatory or infectious diseases where increased tissue damage or pathology is observed.

The onset and incidence of autoimmune diabetes is affected by sex hormones (40, 41). Interestingly, our transcriptomic analysis suggested that some sex hormone related pathways

were affected by the presence or absence of LAG3 on Treg cells (table S1). This may partially explain the differences observed between female and male NOD mice that had *Lag3*-deficient Treg cells, suggesting that there may be additional non-canonical roles for LAG3 in modulating immune responses.

The impact of inhibitory receptors on Treg cell function and maintenance has been controversial. It was reported that the absence of PD1 on Treg cells led to generation of ex-Foxp3 T cells (42). However, Foxp3 stability was maintained in the absence of LAG3 on Treg cells, suggesting distinct pathways are regulated by LAG3 in Treg cells. While our observations here do not preclude a role for LAG3 as a mechanism of Treg suppression (11–13, 15), our data clearly point to a dominant role for LAG3 in limiting Treg cell maintenance and proliferation. This may, in part, be mediated by the Foxp3 co-repressor Eos that is required for Treg cell maintenance (32, 33). Indeed, there seemed to be a direct correlation between Eos expression and Treg cell proliferation, as both were higher following stimulation of *Lag3*-deficient Treg cells, and overexpression or knockdown of Eos resulted in analogous alterations in Treg cell proliferation (Fig. 5). Furthermore, enhanced IL2/STAT5 signaling has been clearly shown to promote Treg cell maintenance and survival (22, 35, 36, 38, 39). LAG3 appears to limit this pathway, thereby having a global impact on Treg cell function. Future studies may shed light on two further questions: (1) Do other inhibitory receptors promote or limit Treg cell function, proliferation and/or survival? (2) Do these inhibitory receptors impact these Treg cell parameters using comparable or distinct mechanisms?

These observations highlight the differential impact of LAG3 modulation on different T cell populations *in vivo*, where LAG3 modulation alleviates or exacerbates disease dependent on whether Treg or Tconv cells are targeted. The impact of losing LAG3 on Treg cells leads to enhanced immune suppression and therefore may offset the effect of blocking the LAG3 pathway in conventional T cells. Indeed, one wonders if this might underlie the lack of efficacy observed on tumor growth with LAG3 blockade (9). These findings may also apply to other inhibitory receptors (4, 14), whose intrinsic effect on Treg cells might have been previously overlooked. Our findings may have clinical relevance as patients who fail to respond to checkpoint blockade immunotherapy may do so because it has a greater impact on promoting Treg cell function than mitigating Tconv cell exhaustion. Thus the differential impact of immunotherapy may be modulated by the Tconv:Treg cell ratio and/or IR expression on different T cell subsets. Given that anti-LAG3 has entered phase I clinical trials for multiple tumor types with the goal to enhance the efficacy of PD1 blockade, we should consider the differential impact this might have on Treg cell function and survival versus Tconv cell exhaustion, and also strategies that specifically target checkpoints on Tconv or Treg cells to boost anti-tumor immunity or mitigate autoimmunity, respectively.

MATERIALS AND METHODS

Mice and study design

NOD/ShiLtJ (stock# 001976), Thy1.1 (stock# 004483) and BDC2.5 (stock# 004460) NOD mice were purchased from Jackson Laboratories. Foxp3^{Cre-GFP} NOD mice were obtained from J.A. Bluestone (43). *Lag3*^{-/-} C57BL/6 mice were obtained from Y.H. Chen with

permission from C. Benoist and D. Mathis and bred onto an NOD background with 100% NOD as determined by single nucleotide polymorphism microsatellite analysis (8, 44). Cd4^{Cre}.NOD mice were obtained from A. Chervonsky.

All animal experiments were performed in American Association for the Accreditation of Laboratory Animal Care-accredited, specific-pathogen-free facilities in Animal Resource Center [St Jude Children's Research Hospital (SJCRH)] and Division of Laboratory Animal Resources [University of Pittsburgh School of Medicine (UPSOM)]. Animal protocols were approved by the Institutional Animal Care and Use Committees (IACUC) of SJCRH and UPSOM. Mice of different groups were co-housed and randomly assigned to any analyses. Ten to twenty mice per group were used in diabetes incidence studies and followed up to 30wk-of-age. Three to five age-matched female mice per group were used in each analytical experiment, and two to four independent experiments were repeated. Three 8wk-of-age female mice per group were pooled and used in RNAseq analyses, and three independent experiments were repeated. The genotypes were not blinded, except for the insulinitis scoring. All data points were presented.

Generation of *Lag3*^{LL-YFP} mice

A 5.7 kb *XbaI-SaII* fragment (5' arm of homology) corresponding to exon 6 and the intronic region between exon 5 and 6 and a 4.1 kb *Clal-EcoRI* fragment (3' arm of homology) containing the polyA site (pA) were generated by PCR from C57BL/6J genomic DNA and cloned into pSP73. A fragment corresponding to exon 7 (containing the CP cleavage site and flanked by loxP sites) and exon 8 was inserted between the two homologous arms. An IRES-YFP fragment was inserted between *Lag3* stop codon and the pA. Just after the pA, a frt-flanked neomycin positive selection cassette (Frt-Neo) was inserted. To increase the frequency of homologous recombination and reduce non-specific integration, a diphtheria toxin cassette (DT-A) was cloned upstream of the 5' homologous arm. The resulting plasmid was linearized with *SspI* and electroporated into E14 ES cells. Following selection with G418, resistant clones were screened by Southern blot analysis, sequenced, injected into blastocysts and the resulting chimeras bred to C57BL/6J for germline transmission. The mice were backcrossed 12 generations onto the NOD background and tested by microsatellite analysis. All 20 *Idd* loci were covered by 144 SNPs in the microsatellite test (45), and all the tested SNPs were NOD.

Measurement of diabetes and insulinitis

Diabetes and insulinitis were assessed as previously described (8, 46). Briefly, diabetes incidence was monitored weekly by testing for the presence of glucose in the urine by Diastix (Bayer). Mice positive by Diastix were then bled and tested with a Breeze2 glucometer (Bayer). Mice were considered diabetic if the blood glucose level was > 400 mg/dl.

Pancreata were embedded in paraffin block and cut at 4μm-thick sections at 150μm step sections and stained with H&E. Pancreata collected at SJCRH were processed at the Veterinary Pathology Core of SJCRH, and pancreata collected at UPSOM were repeated in the same way at HISTO-SCIENTIFIC Research Laboratories (HSRL Inc.). An average of

60–80 islets per mouse were scored in a blinded manner. Two methods of insulinitis measurement were used as previously (46).

Islet isolation and lymphocyte preparation

Islets were isolated as described previously (26). Briefly, the pancreata were perfused with 3mL of collagenase type 4 (Worthington) through the pancreas duct and incubated in 3mL of collagenase (600 U/mL in HBSS with 10% FBS) at 37°C water bath for 30min. The pancreata were then distributed and washed twice with HBSS (Corning) with 10% FBS. The islets were picked under a dissecting microscope, distributed with 1mL of cell dissociation buffer (life technology) and incubated at 37°C for 15min with vortexing every 5min. Following a final wash, the cells were resuspended, counted and used.

Antibodies and flow cytometry

Single cell suspensions were stained with antibodies against CD4 (clone# GK1.5, Biolegend), CD8 β (clone# YTS156.7.7, Biolegend; clone# H35-17.2, eBioscience), TCR β (clone# H57-597, Biolegend), V β 4 (clone# KT4, BD Biosciences), Thy1.1 (clone# OX-7, Biolegend), Thy1.2 (clone# 30-H12, Biolegend), CD45RB (clone# C363-16A, Biolegend), CD44 (clone# IM7, Biolegend), CD62L (clone# MEL-14, Biolegend), CD25 (clone# PC61, Biolegend), LAG3 (clone# 4-10-C9, made in house), Foxp3 (clone# FJK-16s, eBioscience; clone# 150D, Biolegend), Eos (clone# ESB7C2, eBioscience), Helios (clone# 22F6, Biolegend), Ki67 (clone# B56, BD Biosciences), BrdU (clone# Bu20a, Biolegend), Bcl2 (clone# BCL/10C4, Biolegend), TNF α (clone# MP6-XT22, Biolegend), IFN γ (clone# XMG1.2, Biolegend), IL2 (clone# JES6-5H4, Biolegend), IL4 (clone# 11B11, eBioscience), IL17A (clone# TC11-18H10.1, Biolegend), GATA3 (clone# TWAJ, eBioscience), ROR γ t (clone# B2D, eBioscience), PD1 (clone# RMP1-30, Biolegend), TIM3 (clone# RMT3-23, Biolegend), TIGIT (clone# GIGD7, eBioscience), KLRG1 (clone# 2F1, eBioscience), ICOS (clone# C398.4A, Biolegend), phospho-Stat5 (Clone# C71E5, Cell Signaling).

Surface staining was performed on ice for 15min. For cytokine expression analysis, cells were activated with 0.1 μ g/mL PMA (Sigma) and 0.5 μ g/mL Ionomycin (Sigma) in RPMI containing 10% FBS and Monensin (eBioscience) for 5hr. For intracellular staining of cytokines and transcription factors, cells were stained with surface markers, fixed in Fix/Perm buffer (eBioscience) for 0.5–2hr, washed in permeabilization buffer (eBioscience) twice and stained intracellular factors in permeabilization buffer for 30min on ice. For phosphoprotein staining, cells were fixed with 1.6% PFA (Alfa Aesar) at 37°C for 15min, permeabilized with ice-cold Methanol for 1hr, and stained on ice for 1hr. For BrdU incorporation analysis, mice were injected with 2mg BrdU (Sigma) in PBS intraperitoneally 8hr ahead of sacrifice. After transcription factor staining, cells were incubated in Cytofix/Cytoperm buffer (BD Biosciences) at room temperature for 10min, washed with PermWash buffer (BD Biosciences), treated with 650U/mL DNase I (Sigma) at 37°C for 30min, and stained with anti-BrdU antibody in PermWash buffer for 30min at room temperature. The chromogranin A (BDC2.5 mimotope) tetramer for CD4⁺ T cells (AHHPIWARMDA/A^{g7}) and IGRP (NRV-v7 mimotope) tetramer for CD8⁺ T cells (KYNKANVFL/H-2K^d) were obtained from NIH Tetramer Core Facility, and cells were stained in RPMI containing 10% FBS at room temperature for 40min. Cells were sorted on Aria II (BD Biosciences) or

analyzed on Fortessa (BD Biosciences), and data analysis was performed on FlowJo Version 9 (Tree Star).

Micro-Suppression assay

Splenic TCR β ⁺CD4⁺CD45RB⁺GFP⁻ cells were sorted as responder cells and labeled with CellTrace Violet (life technology). T cell-depleted whole splenocytes were treated with 2 μ g/ml mytomycin C (Sigma) at 37°C for 30min, washed three times with PBS, and then used as antigen presenting cells (APCs). Responder cells (4 $\times$ 10³), APCs (8 $\times$ 10³), and different concentrations of Treg cells were activated with 2 μ g/ml anti-CD3 (Biolegend) in a 96-well round bottom plate with 100ul RPMI for 3 days. Suppression was calculated as previously described (47). Briefly, cells were acquired by BD Fortessa, and the division index (DI) of responder cells was analyzed using FlowJo based on the division of CellTrace Violet. Suppression was then calculated with the formula %Suppression = (1-DI_{Treg}/DI_{Ctrl}) $\times$ 100% (DI_{Treg} stands for the division index of responder cells with Treg cells, and DI_{Ctrl} stands for the division index of responder cells activated without Treg cells).

Gene expression profiling by RNAseq and bioinformatic analyses

Treg cells (5 $\times$ 10³) were sorted from three pooled mice of each group and cDNAs were prepared using the SMATer® Ultra™ Low Input RNA Kit for Sequencing - v3 following the user manual (Clontech Laboratories). Sequencing libraries were prepared using Nextera XT DNA Library Preparation kit (Illumina), normalized at 2nM using Tris-HCl (10mM, pH 8.5) with 0.1% Tween20, diluted and denatured to a final concentration of 1.8nM using the Illumina Denaturing and Diluting libraries for the NextSeq 500 protocol Revision D (Illumina). Cluster generation and 75bp paired-end dual-indexed sequencing was performed on Illumina NextSeq 500 system.

The raw reads of RNA sequencing were aligned to the mm10 genome using TopHat and counts were computed relative to the RefSeq transcript annotation file provided in the cufflinks suite (48, 49). Genes whose mean count value (computed in log₂ space) was below 32 (5 in log₂ space) were removed from further processing leaving 10371 total genes. The counts were analyzed for differential expression using DESeq2 with a GC content and length dependent offset computed by cqn R package (50, 51).

Pathway analysis

We performed geneset analysis using the Wilcoxon rank-sum test on the differential expression statistic (Wald statistic for the Negative Binomial coefficient) computed from with the DESeq2 package. Significance was assessed with a parametric *p*-value calculation followed by multiple hypothesis correction as well as sample permutation tests. Since there are three replicates of islet Treg samples of each genotype, there are ten possible ways to divide those into two equal groups, and one of these corresponds to the correct grouping leaving 9 remaining permutations. Pathways that were significant at FDR of 0.2 but were not significant in any of the possible permutation tests were reported. Principle component analysis was performed using the “prcomp” R function on the log₂ transformed normalized counts produced by the DESeq2 “counts” function with “normalized=T”.

Comparisons with *Ikzf4*-knockdown dataset

We retrieved processed data from the GEO accession GSE17166. As this dataset had no replicates we used fold change between the Eos siRNA and control siRNA as a reference. Genes that had expression levels less than $\log_2$ (intensity) of 5 as well as genes that were affected more than 2-fold by the control siRNA were excluded from the analysis. The significance of the association between the two transcriptional signatures was assessed using a Chi-squared test on the contingency table summarizing the number of up- or down-regulated genes in si-*Ikzf4* Treg cells and intra-islet Treg cells.

Treg expansion and adoptive transfer

Splenic TCR β^+ CD4 $^+$ GFP $^+$ CD45RB $^{\text{low}}$ cells (Treg cells) were sorted and activated with 0.1 $\mu\text{g/mL}$ PMA (Sigma) and 0.5 $\mu\text{g/mL}$ Ionomycin (Sigma) with 500U/mL hIL2 (Prometheus) for 2 days, and then expanded for another 3 days with hIL2. WT and *Lag3*-deficient Treg cells were mixed at equal ratio and 2×10^6 total Treg cells were co-transferred into 6–8 wk-of-age WT NODs. Treg recipients were sacrificed and analyzed 4 days post-transfer.

Ikzf4 overexpression and knockdown in Treg cells

Human *IKZF4* ORF was amplified from *IKZF4*-pMIG construct (obtained from C. Benoist (31)) using primers (forward: 5'-CGC GGC TCT AGA TCT GCC AGC ATG CAT ACA CCA CCC GCA CTC C, reverse: 5'-CCT TCC ATC CCT CGA GCT AGC CCA CCT TAT GCT CCC CC), cut with *BglIII* and *XhoI* restriction enzymes, and ligated into the pMI-Ametrine retroviral vector. Murine *Ikzf4* targeting shRNA (3'-TCC AGA AAG AGG ATG CGG CAG T, 5'-CCT GCC GCA TCC TCT TTC TGG A, loop-TAG TGA AGC CAC AGA TGT A) and non-targeting control (3'-TAA CCT ATA AGA ACC ATT ACC A, 5'-CGG TAA TGG TTC TTA TAG GTT A, loop-TAG TGA AGC CAC AGA TGT A) retroviral constructs (transOMIC technologies) were cut with *BglIII* and *MluI* restriction enzymes, and inserted with the IRES-Ametrine cassette as a fluorescence reporter.

Sorted splenic Treg cells were activated with $\alpha\text{CD3}/\alpha\text{CD28}$ dynabeads (Invitrogen) and 500U/mL IL2 for 48hr. Plat-E cells (obtained from H. Chi) were transiently transfected by retroviral vector along with pCL-Eco helper plasmid (obtained from H. Chi). Viral supernatant was harvested 36hr after transfection of Plat-E cells, and then used for spin transduction of activated Treg cells with 6 $\mu\text{g/mL}$ polybrene (Sigma) at 2000rpm for 1hr. Transduced Treg cells were sorted 48hr post transduction, rested for 72hr, and then re-stimulated with 0.1 $\mu\text{g/mL}$ PMA and 0.5 $\mu\text{g/mL}$ Ionomycin with 500U/mL hIL2 for another 48hr. 10 $\mu\text{g/mL}$ BrdU was pulsed into Treg culture media 2hr prior to the staining.

Statistical analyses

Experiments were pooled for statistical analyses using Prism Version 7 (GraphPad). The log-rank test was applied to Kaplan-Meier survival function estimates to determine the statistical significance of differences in diabetes incidence between experimental groups. The Fisher's LSD test was applied to one-way ANOVA to determine the statistical significance in the *Ikzf4* overexpression or knockdown experiments. The nonparametric Mann-Whitney test was used in all other instances.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank J.A. Bluestone (UCSF) for Foxp3^{Cre-GFP}.NOD mice, D. Mathis and C. Benoist for *Lag3*^{-/-} mice and *IKZF4*-pMIG construct, A. Chervonsky for Cd4^{Cre}.NOD mice, P. Murray for the ES cells, H. Chi for the Plat-E cells and pCL-Eco plasmid, and the NIH Tetramer Core Facility (contract HHSN272201300006C) for tetramers. The authors also wish to thank K. Forbes, A. Castellaw, and E.A. Brunazzi for maintenance, breeding, and genotyping of mouse colonies; T. Benos and W. Chen for additional advice regarding computational analysis of the RNAseq data; D. Sawant for assistance with RNA sequencing library preparation; A. Ferguson for assistance with NextGen sequencing; P. Brindle and L. Kasper for technical advice on targeting construct design and ES cell culture; and A. Visperas and A.H. Herrada for advice. The authors also wish to thank R. Cross and G. Lennon (SJCRH) and H. Shen, D. Falkner and A. Yates (UP) for cell sorting; the staff of Animal Resource Center (SJCRH) and Division of Laboratory Animal Resources (UP) for animal husbandry; the Veterinary Pathology Core (SJCRH) for histological preparation; and Genomics Core (UP) for library quantification.

Funding

This work was supported by the National Institutes of Health (R01 DK089125 and P01 AI108545 to D.A.A.V.), NCI Comprehensive Cancer Center Support CORE grant (CA21765 and CA047904, to D.A.A.V.), and ALSAC (to D.A.A.V.).

REFERENCES AND NOTES

1. Zhang Q, Vignali DA. Co-stimulatory and Co-inhibitory Pathways in Autoimmunity. *Immunity*. 2016; 44:1034–1051. [PubMed: 27192568]
2. Callahan MK, Postow MA, Wolchok JD. Targeting T Cell Co-receptors for Cancer Therapy. *Immunity*. 2016; 44:1069–1078. [PubMed: 27192570]
3. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell*. 2015; 27:450–461. [PubMed: 25858804]
4. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. *Immunity*. 2016; 44:989–1004. [PubMed: 27192565]
5. Workman CJ, Vignali DA. The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. *Eur J Immunol*. 2003; 33:970–979. [PubMed: 12672063]
6. Workman CJ, Cauley LS, Kim IJ, Blackman MA, Woodland DL, Vignali DA. Lymphocyte activation gene-3 (CD223) regulates the size of the expanding T cell population following antigen activation in vivo. *J Immunol*. 2004; 172:5450–5455. [PubMed: 15100286]
7. Workman CJ, Vignali DA. Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). *J Immunol*. 2005; 174:688–695. [PubMed: 15634887]
8. Bettini M, Szymczak-Workman AL, Forbes K, Castellaw AH, Selby M, Pan X, Drake CG, Korman AJ, Vignali DA. Cutting edge: accelerated autoimmune diabetes in the absence of LAG-3. *J Immunol*. 2011; 187:3493–3498. [PubMed: 21873518]
9. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, Bettini ML, Gravano DM, Vogel P, Liu CL, Tansombatvisit S, Grosso JF, Netto G, Smeltzer MP, Chaux A, Utz PJ, Workman CJ, Pardoll DM, Korman AJ, Drake CG, Vignali DA. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res*. 2012; 72:917–927. [PubMed: 22186141]
10. Grosso JF, Kelleher CC, Harris TJ, Maris CH, Hipkiss EL, De Marzo A, Anders R, Netto G, Getnet D, Bruno TC, Goldberg MV, Pardoll DM, Drake CG. LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. *J Clin Invest*. 2007; 117:3383–3392. [PubMed: 17932562]
11. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, Hipkiss EL, Ravi S, Kowalski J, Levitsky HI, Powell JD, Pardoll DM, Drake CG, Vignali DA. Role of LAG-3 in regulatory T cells. *Immunity*. 2004; 21:503–513. [PubMed: 15485628]

12. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, Guo B, Herbert DR, Bulfone A, Trentini F, Di Serio C, Bacchetta R, Andreani M, Brockmann L, Gregori S, Flavell RA, Roncarolo MG. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med.* 2013; 19:739–746. [PubMed: 23624599]
13. Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, Flores M, Li N, Schweighoffer E, Greenberg S, Tybulewicz V, Vignali D, Clynes R. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol.* 2008; 180:5916–5926. [PubMed: 18424711]
14. Schildberg FA, Klein SR, Freeman GJ, Sharpe AH. Coinhibitory Pathways in the B7-CD28 Ligand-Receptor Family. *Immunity.* 2016; 44:955–972. [PubMed: 27192563]
15. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol.* 2008; 8:523–532. [PubMed: 18566595]
16. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell.* 2008; 133:775–787. [PubMed: 18510923]
17. Delgoffe GM, Woo SR, Turnis ME, Gravano DM, Guy C, Overacre AE, Bettini ML, Vogel P, Finkelstein D, Bonnevier J, Workman CJ, Vignali DA. Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. *Nature.* 2013; 501:252–256. [PubMed: 23913274]
18. Feuerer M, Shen Y, Littman DR, Benoist C, Mathis D. How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets. *Immunity.* 2009; 31:654–664. [PubMed: 19818653]
19. Bailey-Bucktrout SL, Martinez-Llordella M, Zhou X, Anthony B, Rosenthal W, Luche H, Fehling HJ, Bluestone JA. Self-antigen-driven activation induces instability of regulatory T cells during an inflammatory autoimmune response. *Immunity.* 2013; 39:949–962. [PubMed: 24238343]
20. Depis F, Kwon HK, Mathis D, Benoist C. Unstable FoxP3+ T regulatory cells in NZW mice. *Proc Natl Acad Sci U S A.* 2016; 113:1345–1350. [PubMed: 26768846]
21. Komatsu N, Okamoto K, Sawa S, Nakashima T, Oh-hora M, Kodama T, Tanaka S, Bluestone JA, Takayanagi H. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. *Nat Med.* 2014; 20:62–68. [PubMed: 24362934]
22. Tang Q, Adams JY, Penaranda C, Melli K, Piaggio E, Sgouroudis E, Piccirillo CA, Salomon BL, Bluestone JA. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity.* 2008; 28:687–697. [PubMed: 18468463]
23. Reed JC, Herold KC. Thinking bedside at the bench: the NOD mouse model of T1DM. *Nat Rev Endocrinol.* 2015; 11:308–314. [PubMed: 25623120]
24. Li N, Wang Y, Forbes K, Vignali KM, Heale BS, Saftig P, Hartmann D, Black RA, Rossi JJ, Blobel CP, Dempsey PJ, Workman CJ, Vignali DA. Metalloproteases regulate T-cell proliferation and effector function via LAG-3. *EMBO J.* 2007; 26:494–504. [PubMed: 17245433]
25. Li N, Workman CJ, Martin SM, Vignali DA. Biochemical analysis of the regulatory T cell protein lymphocyte activation gene-3 (LAG-3; CD223). *J Immunol.* 2004; 173:6806–6812. [PubMed: 15557174]
26. Lennon GP, Bettini M, Burton AR, Vincent E, Arnold PY, Santamaria P, Vignali DA. T cell islet accumulation in type 1 diabetes is a tightly regulated, cell-autonomous event. *Immunity.* 2009; 31:643–653. [PubMed: 19818656]
27. Marrack P, Kappler J. Control of T cell viability. *Annu Rev Immunol.* 2004; 22:765–787. [PubMed: 15032596]
28. Tauro S, Nguyen P, Li B, Geiger TL. Diversification and senescence of Foxp3+ regulatory T cells during experimental autoimmune encephalomyelitis. *Eur J Immunol.* 2013; 43:1195–1207. [PubMed: 23436224]
29. Kornete M, Sgouroudis E, Piccirillo CA. ICOS-dependent homeostasis and function of Foxp3+ regulatory T cells in islets of nonobese diabetic mice. *J Immunol.* 2012; 188:1064–1074. [PubMed: 22227569]
30. D'Alise AM, Auyeung V, Feuerer M, Nishio J, Fontenot J, Benoist C, Mathis D. The defect in T-cell regulation in NOD mice is an effect on the T-cell effectors. *Proc Natl Acad Sci U S A.* 2008; 105:19857–19862. [PubMed: 19073938]

31. Fu W, Ergun A, Lu T, Hill JA, Haxhinasto S, Fassett MS, Gazit R, Adoro S, Glimcher L, Chan S, Kastner P, Rossi D, Collins JJ, Mathis D, Benoist C. A multiply redundant genetic switch ‘locks in’ the transcriptional signature of regulatory T cells. *Nat Immunol.* 2012; 13:972–980. [PubMed: 22961053]
32. Pan F, Yu H, Dang EV, Barbi J, Pan X, Grosso JF, Jinasena D, Sharma SM, McCadden EM, Getnet D, Drake CG, Liu JO, Ostrowski MC, Pardoll DM. Eos mediates Foxp3-dependent gene silencing in CD4⁺ regulatory T cells. *Science.* 2009; 325:1142–1146. [PubMed: 19696312]
33. Sharma MD, Huang L, Choi JH, Lee EJ, Wilson JM, Lemos H, Pan F, Blazar BR, Pardoll DM, Mellor AL, Shi H, Munn DH. An inherently bifunctional subset of Foxp3⁺ T helper cells is controlled by the transcription factor eos. *Immunity.* 2013; 38:998–1012. [PubMed: 23684987]
34. Ohkura N, Hamaguchi M, Morikawa H, Sugimura K, Tanaka A, Ito Y, Osaki M, Tanaka Y, Yamashita R, Nakano N, Huehn J, Fehling HJ, Sparwasser T, Nakai K, Sakaguchi S. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity.* 2012; 37:785–799. [PubMed: 23123060]
35. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol.* 2005; 6:1142–1151. [PubMed: 16227984]
36. D’Cruz LM, Klein L. Development and function of agonist-induced CD25⁺Foxp3⁺ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol.* 2005; 6:1152–1159. [PubMed: 16227983]
37. Gonzalez A, Katz JD, Mattei MG, Kikutani H, Benoist C, Mathis D. Genetic control of diabetes progression. *Immunity.* 1997; 7:873–883. [PubMed: 9430232]
38. Johnson MC, Garland AL, Nicolson SC, Li C, Samulski RJ, Wang B, Tisch R. beta-cell-specific IL-2 therapy increases islet Foxp3⁺Treg and suppresses type 1 diabetes in NOD mice. *Diabetes.* 2013; 62:3775–3784. [PubMed: 23884888]
39. Grinberg-Bleyer Y, Baeyens A, You S, Elhage R, Fourcade G, Gregoire S, Cagnard N, Carpentier W, Tang Q, Bluestone J, Chatenoud L, Klatzmann D, Salomon BL, Piaggio E. IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. *J Exp Med.* 2010; 207:1871–1878. [PubMed: 20679400]
40. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, von Bergen M, McCoy KD, Macpherson AJ, Danska JS. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science.* 2013; 339:1084–1088. [PubMed: 23328391]
41. Yurkovetskiy L, Burrows M, Khan AA, Graham L, Volchkov P, Becker L, Antonopoulos D, Umesaki Y, Chervonsky AV. Gender bias in autoimmunity is influenced by microbiota. *Immunity.* 2013; 39:400–412. [PubMed: 23973225]
42. Zhang B, Chikuma S, Hori S, Fagarasan S, Honjo T. Nonoverlapping roles of PD-1 and FoxP3 in maintaining immune tolerance in a novel autoimmune pancreatitis mouse model. *Proc Natl Acad Sci U S A.* 2016; 113:8490–8495. [PubMed: 27410049]
43. Zhou X, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT, Bluestone JA. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J Exp Med.* 2008; 205:1983–1991. [PubMed: 18725525]
44. Miyazaki T, Dierich A, Benoist C, Mathis D. Independent modes of natural killing distinguished in mice lacking Lag3. *Science.* 1996; 272:405–408. [PubMed: 8602528]
45. Steward CA, Gonzalez JM, Trevanion S, Sheppard D, Kerry G, Gilbert JG, Wicker LS, Rogers J, Harrow JL. The non-obese diabetic mouse sequence, annotation and variation resource: an aid for investigating type 1 diabetes. *Database (Oxford).* 2013; 2013:bat032. [PubMed: 23729657]
46. Leiter EH. The NOD mouse: a model for insulin-dependent diabetes mellitus. *Curr Protoc Immunol.* 2001; 19. Chapter 15, Unit 15.
47. McMurchy AN, Levings MK. Suppression assays with human T regulatory cells: a technical guide. *Eur J Immunol.* 2012; 42:27–34. [PubMed: 22161814]
48. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics.* 2009; 25:1105–1111. [PubMed: 19289445]

49. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol.* 2010; 28:511–515. [PubMed: 20436464]
50. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014; 15:550. [PubMed: 25516281]
51. Hansen KD, Irizarry RA, Wu Z. Removing technical variability in RNA-seq data using conditional quantile normalization. *Biostatistics.* 2012; 13:204–216. [PubMed: 22285995]

Figure 1. LAG3 is upregulated on intra-islet Treg cells

(A) WT NOD Treg expression profiles shown in XY dot plots. Values were averaged from independent triplicates (female, 8 wk-of-age). Labels denote IR genes that are significantly up-regulated (Red) or down-regulated (Blue) in intra-islet Treg cells compared with periphery Treg cells.

(B) Representative histograms of LAG3 expression on cell surface of CD8⁺, CD4⁺Foxp3⁻, and CD4⁺Foxp3⁺ T cells in female WT NODs at 8 wk-of-age.

(C) Percent of LAG3⁺ Treg cells (top) and MFI (fold change relative to isotype control) of LAG3 on Treg cells (bottom) in WT NODs at 6, 8, and 10 wk-of-age (n=5–6, females). ndLN, non-draining lymph node; PLN, pancreatic lymph node; MFI, mean fluorescence

intensity. Data represent mean $\pm$ SEM. Statistic significance was determined by comparison of Islet and ndLN at each time point.

(D) Proportion of CD4⁺Foxp3⁻, CD8⁺, and CD4⁺Foxp3⁺ cells in intra-islet LAG3⁺ T cells. Nonparametric Mann-Whitney test was used in (C). ns, not significant; * $P < 0.05$, ** $P < 0.01$.

Figure 2. Loss of LAG3 on Treg cells results in reduced autoimmune diabetes

(A) Schematic of WT and *Lag3*^{L/L-YFP} loci.

(B) Diabetes onset and incidence monitored in *Lag3*^{L/L-YFP}Cd4^{Cre}.NOD females and co-caged littermate controls.

(C) Diabetes onset and incidence monitored in *Lag3*^{L/L-YFP}Foxp3^{Cre-GFP}.NOD females (top) and males (bottom) together with co-caged littermate controls.

(D) Histological assessment of insulinitis performed in female and male *Lag3^{L/L}-YFP^{Foxp3^{Cre}-GFP}.NOD* mice together with co-caged controls at 6 and 10 wk-of-age (n=4–7). Horizontal black bars indicate the mean. Error bars indicate SEM. The log-rank test was applied to Kaplan-Meier survival function estimates to determine the statistical significance in (B–C), and nonparametric Mann-Whitney test was used in (D). ns, not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Figure 3. LAG3 alters the Treg transcriptome

(A) Heatmap of differentially expressed genes in *Lag3*-deficient versus WT ndLN and intra-islet Treg cells. Fold change in gene expression was relative to WT Treg cells from ndLN, and shown as the mean of independent triplicates (female, 8 wk-of-age). The gene dendrogram was calculated based on standard Euclidean distance mean linkage clustering and rotated to sort values in islet *Lag3^{L/L}-YFP⁺Foxp3^{Cre-GFP}.NOD* Treg cells. The following gene sets were used for comparison: Treg signature from ref. (30, 31); IL2-Stat5 targeted genes from mSigDB (HALLMARK_IL2_STAT5_SIGNALING); si-*Ikzf4* Treg cells (Treg

cells knockdown with *Ikzf4* siRNA) and si-RL Treg cells (Treg cells knockdown with control siRNA) from ref. (32).

(B) Scatter plot of the Eos targeted genes (32) in *Lag3*-deficient versus WT Treg expression profiles.

(C) Differentially expressed IL2-STAT5 targeted genes shown in XY dot plots. Labels denote genes from mSigDB HALLMARK_IL2_STAT5_SIGNALING that are significantly up-regulated (Red) or down-regulated (Blue) in *Lag3*-deficient Treg cells compared to WT Treg cells.

(D) Barcode plots depicting the enrichment of genes in the mSigDB WIERENGA_STAT5_TARGETS_UP and HALLMARK_IL2_STAT5_SIGNALING pathways. Statistics denotes the Wald statistic used to test the significance of coefficients in a negative binomial generalized linear model.

Figure 4. LAG3 intrinsically limits Treg cell proliferation

(A) Schematic of Treg co-transfer experiment.

(B) Representative plots of Thy1.1⁺ (WT: blue) and Thy1.2⁺ (*Lag3*-deficient: red) Treg cells (gated on CD4⁺Vβ4⁺) in NOD recipients (grey).

(C) Proportion of WT and *Lag3*-deficient Treg cells in the islets and LNs assessed by the percent of Thy1.1⁺ and Thy1.2⁺ in CD4⁺Vβ4⁺ cells post-transfer (n=17, three independent experiments; female mice). Data presented as mean ± SEM.

(D–I) Flow cytometric analysis of markers shown. Horizontal black bars indicate the mean (D–E: n=17, three independent experiments; F–I: n=12, two independent experiments; all female mice).

Nonparametric Mann-Whitney test was used in (C–I). ns: not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 5. LAG3 limits Treg proliferation through Eos pathway

(A – B) Eos expression (top) and BrdU incorporation (bottom) assessed in activated Treg cells post knockdown (A, four independent experiments) or overexpression (B, three independent experiments) of *Ikzf4*. WT, Foxp3^{Cre-GFP}.NOD; KO, *Lag3*^{Δ/Δ}-YFP^{Foxp3}^{Cre-GFP}.NOD; NTC, non-targeting control vector; EV, empty vector pMIA. Horizontal black bars indicate the mean.

The Fisher's LSD test was applied to one-way ANOVA to determine the statistical significance. ns: not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.