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Cutting Edge: Accelerated Autoimmune Diabetes in the Absence of LAG-3

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Lymphocyte activation gene-3 (LAG-3; CD223) is a CD4 homolog that is required for maximal regulatory T cell function and for the control of CD4+ and CD8+ T cell homeostasis. Lag3−/− NOD mice developed substantially accelerated diabetes with 100% incidence. Adoptive transfer experiments revealed that LAG-3 was primarily responsible for limiting the pathogenic potential of CD4+ T cells and, to a lesser extent, CD8+ T cells. Lag3−/− mice exhibited accelerated, invasive insulitis, corresponding to increased CD4+ and CD8+ T cell islet infiltration and intraislet proliferation. The frequencies of islet Ag-reactive chromogranin A-specific CD4+ T cells and islet specific glucose-6-phosphatase–specific CD8+ T cells were significantly increased in the islets of Lag3−/− mice, suggesting an early expansion of pathogenic clones that is normally restrained by LAG-3. We conclude that LAG-3 is necessary for regulating CD4+ and CD8+ T cell function during autoimmune diabetes, and thus may contribute to limiting autoimmunity in disease-prone environments. The Journal of Immunology, 2011, 187: 3493–3498.

Type 1 diabetes (T1D) is a chronic autoimmune disorder driven primarily by CD4+ T cells in concert with CD8+ T, B, and NK cells (1). Autoimmune diabetes in NOD mice, a model for T1D in humans (2), is initially associated with cellular infiltration of the pancreatic islets (insulitis). Ultimately, the balance between regulatory and proinflammatory mediators is lost, leading to the destruction of insulin-secreting β cells and subsequent diabetes (2). The importance of regulatory elements in the early prediabetic stages of disease was exposed on regulatory T cell (Treg) ablation, which results in rapid β cell destruction and diabetes onset (3). However, the regulatory mechanisms responsible for limiting catastrophic disease in autoimmune diabetes-prone mice and patients with T1D remain obscure.

Cell-intrinsic negative regulatory molecules, such as CTLA-4, programmed death-1 (PD-1), and lymphocyte activation gene-3 (LAG-3; CD223), mediate immune regulation with overlapping but distinct characteristics. For instance, whereas PD-1 blockade in NOD mice induces rapid diabetes at any age, disease after CTLA-4 blockade is induced only in neonates (4). LAG-3 is a CD4 homolog that binds to MHC class II molecules with high affinity. It regulates T cell proliferation and homeostasis, CD8+ T cell exhaustion, and is required for optimal Treg and NK cell function (5–8). However, Lag3 deletion in C57BL/6 mice resulted in a minor phenotype with no development of spontaneous disease, arguing against a significant role for LAG-3 in controlling autoimmunity (9).

We hypothesized that the function of LAG-3 becomes more critical in autoimmune-prone environments. Although recent studies suggested that autoimmune diabetes is accelerated in Lag3−/− NOD, the mechanistic basis for this increased disease is unknown (10). In our study, we determined the effect of LAG-3 loss or blockade on autoimmune diabetes incidence in NOD mice and asked how this might involve T cells, their functional parameters, and/or the frequency of presumptive pathogenic T cell populations.

Materials and Methods
Mice and diabetes induction
Lag3−/− C57BL/6 mice (9) (obtained from Yueh-Hsiu Chen, Stanford University, Palo Alto, CA, with permission from Christophe Benoist and Diane Mathis, Joslin Diabetes Center, Boston, MA) were bred onto the NOD background for at least 10 generations (100% NOD as determined by single-nucleotide polymorphism microsatellite analysis) or onto the B10.D2 background for at least 10 generations (at Johns Hopkins University). NOD.scid and NOD/ShiLtJ mice were obtained from Jackson Laboratories, bred at St. Jude. In some experiments, diabetes was adoptively transferred by injecting NOD.scid female mice i.v. with 107 splenocytes. All animal experiments were in Association for the Accreditation of Laboratory Animal Care-accredited, Helicobacter-free, murine norovirus-free, specific pathogen-free facilities in the

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Abbreviations used in this article: IGRP, islet specific glucose-6-phosphatase; LAG-3, lymphocyte activation gene-3; PD-1, programmed death-1; PLN, pancreatic lymph node; T1D, type 1 diabetes; Treg, regulatory T cell; WT, wild type.

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st. jude animal resource center following national, state and institutional guidelines. blockade experiments were performed at biologics discovery california, bristol-myers-squibb (milpitas, ca).

measurement of insulitis and diabetes

insulitis and diabetes was assessed as described previously (11, 12). in brief, pancreata of nod mice embedded in paraffin was cut at 4-μm-thick sections at 150-μm step sections and stained with h&e at the st. jude histology core facility. an average of 90–100 islets per mouse was scored in a blinded manner. two methods of insulitis measurement were used. first, perivascular and peri-insulitis were determined based on the percentage of the islets that possessed leukocyte infiltrate using the following metric: no insulitis (normal islet and no infiltration), peri-insulitis (infiltration on edges of islet or 0–25% of islet infiltrated), or insulitis (infiltration of 25–100% of islet) (11). second, a defined insulitis score was determined using the method outlined in current protocols in immunology (13). diabetes incidence was monitored weekly by testing for the presence of glucose in the urine by clinicists (bayer, elk hart, in). mice testing positive by clinicists were then tested with a breez2 glucometer (bayer) for elevated blood glucose levels and were considered diabetic if their blood glucose level was >400 mg/dl.

anti–lag-3 and anti–pd-1 blockade

seven-wk-old female nod mice were treated by i.p. injection with 200 μg chimeric mouse anti–pd-1 (4h2, ig1g1), rat anti-mouse lag-3 (c09b7w, ig1g1), or control murine ig1g1 (mopc 21; bioxcell) on days 0, 4, and 7. mice were tested twice a week for blood glucose levels and were considered diabetic after two consecutive readings >250 mg/dl.

islet isolation, flow cytometry, and sorting

islets were isolated as described previously (12). the chromogranin a (bdc2.2 mimotope) tetramer for cd4+ t cells (ahhpiwarmda/αβ) and the islet specific glucose-6-phosphatase (igrp) (nrp-v7 mimotope) tetramer for cd8+ t cells (kynkanvfl/h-2kβ) were obtained from national institutes of health tetramer core. surface ag and igrp tetramer staining were performed on ice for 30 min. bdc tetramer staining was done for 2 h at 37 °c in cell culture media, in parallel with control cd4 tetramer (lsialhvgfdh/αβ). nrp-v7 tetramer enrichment was performed on pooled peripheral lymphoid organs (excluding pancreatic lymph nodes [plns]) as described previously (14). in brief, pooled organs were labeled with nrp-v7 tetramer, followed by anti-pe macs bead (miltenyi) purification, and the positively selected fraction was counted and analyzed by flow cytometry.

statistical analysis

kaplan–meier test was used to determine significance of diabetes incidence in combination with log-rank test to determine the p value. the mann–whitney nonparametric analysis was used in all other instances to determine significance and the p value.

results and discussion

accelerated diabetes after loss or blockage of lag-3

to assess the potential role of lag-3 in autoimmune, we crossed the lag3+/- mice onto the autoimmune-prone nod background. deletion of lag-3 resulted in rapid diabetes development in both nod females and males, with 100% penetrance at the time when the wild type (wt) female mice exhibited only 15% disease onset; this amounted to 10 and 14 wk earlier onset in females and males, respectively (at 50% incidence; fig. 1a, 1b). diabetes incidence in heterozygous mice was clearly indicative of haploinsufficiency, suggesting that even a partial reduction in lag3 gene expression leads to accelerated disease.

we further assessed whether the defect observed was due to loss of lag-3, rather than disruption of an adjacent locus in the targeted mice, by determining the effect of lag-3 blockade. we also sought to determine whether disruption of lag-3 function in insulin, prediabetic mice caused accelerated diabetes. seven-wk-old nod female mice were given three doses of blocking anti–lag-3 ab and monitored for diabetes induction. anti–pd-1 blockade was included for comparison because such treatment has previously been shown to induce rapid diabetes onset (4). blocking lag-3 in wt mice accelerated disease development by ~8 wk, comparable with the effect seen in the lag3+/- mice and with a similar incidence to anti–pd-1 treatment, albeit with slightly delayed kinetics (fig. 1c). taken together, these data show that lag-3 genetic deletion or blockade substantially accelerates diabetes incidence and onset, suggesting that lag-3 limits disease onset and mitigates an ongoing autoimmune response. this contrasts with blockade of cdta-4 and icos that have a dominant role only at earlier stages of insulitis (<4-wk-old mice) (15).

lag3+/- cd4+ t cells alone can recapitulate the lag3+/- phenotype

because cd4+ and cd8+ t cells can express lag-3 and both cell types are instrumental in driving diabetes development, we assessed the relative contribution of each population in the accelerated diabetes observed (1). because the disease environment in nod mice involves multiple cellular populations, we wanted to assess how the loss of lag-3 affects cd4+ and cd8+ t cells in the context of wt lymphoid populations. we chose to use splenocytes from young prediabetic 5- to 6-wk-old mice so that the experiment was not biased by the possibility that a larger number of islet reactive t cells might
be present in Lag3−/− NOD mice. Splenocytes from 5- to 6-wk-old WT NOD mice were depleted of CD4+ or CD8+ cells and then reconstituted with either Lag3−/− or WT CD4+ or CD8+ cells, respectively, and adoptively transferred into NOD.scid recipients. Reciprocal experiments were also performed where CD4+ or CD8+ T cell-depleted Lag3−/− splenocytes were reconstituted with WT CD4+ or CD8+ T cells, respectively. As a control, depleted Lag3−/− splenocytes were reconstituted with Lag3−/− T cells, and WT splenocytes reconstituted with WT T cells. Importantly, adoptive transfer of splenocytes from Lag3−/− mice into NOD.scid female recipients resulted in accelerated disease compared with WT splenocyte recipients (Fig. 1D).

Interestingly, transfer of Lag3−/− CD4+ T cells in the context of WT splenocytes also resulted in accelerated disease, suggesting that loss of LAG-3 expression from CD4+ T cells is sufficient to recapitulate the accelerated diabetes onset and incidence observed in the Lag3−/− splenocyte recipients (Fig. 1D). Likewise, adoptive transfer of WT CD8+ T cells plus CD8-depleted Lag3−/− splenocytes (i.e., contained Lag3−/− CD8+ T cells) also resulted in accelerated disease (Fig. 1E). These data suggested that Lag3−/− CD4+ T cells are sufficient to drive the accelerated diabetes onset and incidence observed.

To assess the contribution of Lag3−/− CD8+ T cells, we transferred either Lag3−/− CD8+ T cells plus CD8-depleted WT splenocytes or WT CD4+ T cells plus CD4-depleted Lag3−/− splenocytes (i.e., contained Lag3−/− CD8+ T cells). Interestingly, diabetes onset was also accelerated, but in both cases final disease incidence was lower and somewhat comparable with transfer of WT splenocytes (Fig. 1D, 1E). These data suggest that although loss of LAG-3 on CD8+ T cells can lead to accelerated disease onset, this does not translate into increased disease penetrance, inferring that the loss of LAG-3 on CD4+ T cells has a greater impact on disease development.

**Accelerated T cell infiltration in the islets of Lag3−/− mice**

Next, we asked whether the kinetics of islet infiltration mirrored accelerated diabetes development in Lag3−/− NOD mice. Histological analysis revealed accelerated insulitis in Lag3−/− NOD female and male mice starting as early as 4 wk of age (Fig. 2A, 2B). Interestingly, insulitis was accelerated by only a few weeks, unlike diabetes, which was accelerated by 10–14 wk, suggesting that once insulitis is initiated, the β cell destruction rapidly ensues in the absence of Lag-3.

Lag3 is expressed by multiple cell types including CD4+ and CD8+ T cells, NK cells, and plasmacytoid dendritic cells (16). Therefore, we wanted to assess which populations were differentially represented in the pancreatic islets of Lag3−/− NOD mice. We concentrated our analysis on prediabetic 6-wk-old female WT and Lag3−/− mice. However, because of temporal differences in diabetes onset between WT and Lag3−/− mice, in some instances, we also included 10-wk-old WT mice because this is a comparable time point to Lag3−/− mice relative to diabetes onset. The total number of cells infiltrating the islets of 6-wk-old Lag3−/− mice was increased compared with age-matched WT mice but comparable with 10-wk-old WT controls (Supplemental Fig. 1A). There was a significant increase in the frequency and number of CD4+ and CD8+ T cells in the islets of 6-wk-old mice, which were comparable with 10-wk-WT mice (Fig. 2C, 2D). These differences were limited to the islets because this was not observed in the PLNs. Although accelerated islet infiltration was observed in Lag3−/− NOD mice, this appeared to plateau as a comparable percentage and number of CD4+ T cells was observed in the islets of 9- to 10-wk-old WT and Lag3−/− NOD mice, although the latter were hyperglycemic at this time point (Supplemental Fig. 1B). Taken together, these data suggest that islet infiltration is accelerated in Lag3−/− NOD mice, although the extent and constituency of the islet infiltrate at diabetes onset is similar in WT and Lag3−/− NOD mice. Thus, the accelerated disease onset may be because of early, rapid islet infiltration rather than an increase in the final total T cell numbers.
In contrast with CD4+ and CD8+ T cells, there was no significant difference in the frequency of CD4+Foxp3+ Tregs, although a small increase in the numbers was observed (Fig. 2D). Interestingly, the frequency and number of Tregs were significantly reduced in the islets of diabetic Lag3−/− NOD mice (Supplemental Fig. 1B), consistent with the dramatic Treg reduction seen in late-stage disease, which is thought to be due to reduced IL-2 at the site of inflammation (17).

We did not observe any significant increase in the frequency or number of NK cells or plasmacytoid dendritic cells in the islets of Lag3−/− NOD mice (data not shown). These data suggest that the absence of LAG-3 results in a selective increase in CD4+ and CD8+ T cells.

Increased T cell proliferation but not effector function in the absence of LAG-3

We hypothesized that enhanced T cell accumulation in the islets of Lag3−/− NOD mice could be caused by accelerated trafficking and/or increased proliferation. About 5% of CD4+ or CD8+ T cells in the nondraining lymph nodes and PLNs expressed Ki67, a marker associated with cell cycle progression (Fig. 3A). The frequency of Ki67+ CD4+ and CD8+ T cells was augmented in the islets of NOD mice, although this did not seem to increase with age. Interestingly, there was a significant increase in the percentage of Ki67+ CD4+ and CD8+ T cells in Lag3−/− NOD mice. A higher percentage of Ki67+ Foxp3+ Tregs, compared with CD4+ and CD8+ T cells, was observed in all organs examined. However, although the frequency of Ki67+ Tregs was substantially higher in the islets, no discernible increase was observed in Lag3−/− mice. These data suggest that increased intraislet proliferation may contribute to the increased T cell accumulation seen in Lag3−/− NOD mice.

Expression of the homing molecules, CXCR3 chemokine receptor and α4 integrins, have been associated with T cell trafficking into the pancreas (18, 19). A substantially higher percentage of CD4+CD44hi T cells in the PLNs expressed CXCR3 compared with other lymphoid organs (Fig. 3B). Interestingly, the frequency of CXCR3+CD4+CD44hi T cells was significantly higher in the PLNs of Lag3−/− mice. A high percentage of CD8+CD44hi T cells expressed CXCR3 in all the secondary lymphoid organs tested, and this did not differ in the absence of LAG-3 (Supplemental Fig. 1C). Likewise, a high percentage of CD4+ and CD8+ T cells infiltrating the islets expressed the α4 integrin chain, although there was no difference in expression between WT and Lag3−/− mice (Supplemental Fig. 1D). Overall, these data suggest that accelerated islet infiltration in Lag3−/− NOD mice may primarily be caused by an increase in CD4+ T cell homing to the pancreas followed by increased proliferation of CD4+ and CD8+ T cells in the islets.

We next assessed whether the effector function of islet-infiltrating T cells in Lag3−/− mice was altered. There was an increased frequency of CD4+IFN-γ+ and CD8+IFN-γ+ T cells in the islets, compared with secondary lymphoid organs (Fig. 3C). In contrast, minimal differences in the frequency of TNF-α+ and IL-2+ T cells were observed (Supplemental Fig. 1E–H, 1J, 1K). Also, very low percentages of IL-17+ T cells were present in the islets, questioning the contribution of Th17 cells in autoimmune diabetes (Supplemental Fig. 1L). Interestingly, there was no increase in the frequency of effector cytokine-expressing T cells in the islets of Lag3−/− mice compared with WT mice, suggesting that the accelerated disease observed in Lag3−/− mice is due to increased T cell infiltration/retention/proliferation, and not due to increased T cell effector function.

Increased frequency of islet Ag-specific T cells in Lag3−/− NOD mice

Because Lag3−/− NOD mice exhibited earlier and more extensive T cell infiltration into the islets, we questioned whether the PLNs and islets in the Lag3−/− mice had an increased proportion of islet Ag-reactive T cells. We used MHC:peptide tetramers specific for two major autoantigens, chromogranin A and IGRP, recognized by pathogenic CD4+ and CD8+ T cells, respectively, to determine the frequency of potentially pathogenic clones that may be associated with progression to disease (20, 21). WT and Lag3−/− mice exhibited low frequencies of islet Ag tetramer+ T cells within activated CD4+CD44hi and CD8+CD44hi T cell populations in the nonnondraining and draining lymph nodes, but were significantly elevated in the islets, in contrast with nonislet Ag tetramer (Fig. 4A, 4B, Supplemental Fig. 1J). Importantly, there was a 2- to 4-fold increase in the frequency of tetramer+ T cells in the islets of Lag3−/− mice compared with WT controls, suggesting that the loss of LAG-3 may have a

**FIGURE 3.** CD4+ and CD8+ T cells exhibit increased proliferation in the islets of Lag3−/− mice. A, Proliferation was assessed based on intracellular Ki67 staining of CD8+, CD4+Foxp3+ , and CD4+Foxp3− T cells in 6-wk-old Lag3−/− female mice (n = 7) and 6- (n = 10) or 10-wk-old (n = 5) WT female mice (*p = 0.00229). B, CXCR3 surface expression on CD4+ T cells was measured by flow cytometric analysis of 6- to 7-wk-old female Lag3−/− mice (n = 7; *p = 0.0262). C, Intracellular IFN-γ expression was assessed in the organs of 6-wk-old female and 7-wk-old male Lag3−/− and WT mice after 5-h stimulation with PMA and ionomycin (n = 7–10).
selective effect on more diabetogenic clones. This increase is relative to the total number of T cells in the islets and, thus, a preferential expansion of pathogenic clones compared with other infiltrating T cell clones. Thus, the actual number of tetramer+ T cells in the islets of Lag3−/− NOD mice is even greater given the increased number of infiltrating T cells observed.

We then posited that the increased frequency of pathogenic clones in the islets of Lag3−/− NOD mice could be caused by accelerated or preferential pathogenic clone expansion on activation in the PLNs or islets, or increased precursor frequency of autoreactive clones in Lag3−/− NOD mice. To quantify naive precursors in the periphery of Lag3−/− mice, we used a previously published method for quantification of small numbers of endogenous Ag-specific T cells (14). We pooled all peripheral lymphoid organs in individual mice excluding draining PLNs and then used anti-PE MACS beads to enrich for IGRP:H-2Kd-PE tetramer+ CD8+ cells. In concordance with these data, there was no difference in the number of IGRP-specific thymocytes after tetramer enrichment of single-positive CD8+CD3+ thymocytes (data not shown). Unexpectedly, 30% of young (6-wk-old) Lag3−/− NOD mice had slightly increased numbers of activated CD44lo tetramer+ cells in nondenraining peripheral organs, and these cells seemed to accumulate in periphery with age (Fig. 4D, Supplemental Fig. 1M). Thus, we conclude that LAG-3 primarily functions at a local level where its absence causes increased T cell proliferation after Ag stimulation, which leads to the rapid accumulation of self-reactive T cells and accelerated disease.

In conclusion, our data show that LAG-3 plays a critical role in regulation of autoimmune response in NOD mice by selectively inhibiting Ag-reactive T cell infiltration and expansion in the islets, without affecting the effector phenotype of the cells. In the absence of LAG-3, T cells infiltrate NOD pancreatic islets at a younger age and accumulate faster, processes that are associated with increased CXCR3 expression on CD4+ T cells in the PLNs and increased CD4+ and CD8+ T cell proliferation in the islets of Lag3−/− NOD mice. Given that blockade of LAG-3 after onset of islet infiltration in 7-wk-old NOD mice results in accelerated diabetes development, our findings suggest that LAG-3 functions throughout disease development. Previous studies have suggested that LAG-3 has a preferential effect on the proliferative capacity of T cells (5, 7, 8), in contrast with PD-1 and CTLA-4 that appear to affect both proliferation and effector function (8, 22). It should be noted that the only known ligand for murine LAG-3 is MHC class II, to which it binds with high affinity (7, 23, 24). Thus, LAG-3 is likely ligated by the macrophages, dendritic cells, and B cells that are known to be present in the islet infiltrate and express high levels of MHC class II (1). A particularly interesting finding in our study was the apparent preferential expansion of islet Ag-specific, potentially pathogenic T cell populations. Our previous studies have suggested that only islet Ag-specific T cells can enter the islets (12). Thus, we anticipated that the absence of LAG-3 might affect all infiltrating T cells comparably. However, these data suggest that the absence of LAG-3 may have a greater effect on the more pathogenic T cell clones responsible for initiating and/or driving diabetes onset. Interestingly, we also showed that this preferential expansion of potentially pathogenic clones may be limited to the islets, although these cells can accumulate in the periphery at later stages of disease. Our study suggests that further analysis of the role of LAG-3 in modulating autoimmunity is warranted, particularly given that linkage analysis has suggested that single nucleotide polymorphisms in the LAG3 locus may confer susceptibility to multiple sclerosis in human patients (25). Furthermore, the therapeutic enhancement of LAG-3 activity, perhaps via agonistic mAbs, warrants further investigation as a treatment for T1D.
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Disclosures

D.A.A.V. has submitted patents that are pending and is entitled to a share in development. A.J.K. and M.S. are employees of Bristol-Myers-Squibb.

References

Supplementary Data:

**Accelerated autoimmune diabetes in the absence of LAG-3**

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Supplementary Fig. 1. A, Total cell number in the pancreatic LN (PLN) and islets of 6 week old Lag3−/− female mice (n = 29) and 6 (n = 21) or 10 week old (n = 22) wild type female mice (*p < 0.04). B, Percent and total number of CD4+ and CD4+Foxp3+ T cells in older (9-10 week old) mice (n = 4-8; **p<0.002, *p<0.05). Note that all the Lag3−/− NOD mice were hyperglycemic at this time point. Surface expression of homing markers based on flow cytometric analysis of CXCR3 (C) and alpha 4 integrin subunit (D) expression on the surface of CD8+ and CD4+ T cells. E-I, Cytokine expression in CD4+ and CD8+ T cells after 5 hour restimulation with PMA/ionomycin (G and J, n = 7; H and K, n = 10; I, n = 5-7). E and F, representative FACS plots. M, Glucose-6-phosphatase isomerase peptide tetramer staining (control for Fig. 4A) of 6 week old wild type and Lag3−/− female mice (LSIALHVGFDH/I-Ag7) (n = 14-15). Class II tetramer staining was done in complete media for 2 hours at 37°C at 2μg/mL, followed by staining for surface makers at 4°C. Analysis is gated on single cells (SSC-A vs SSC-W), B220−Gr1−CD3+CD8−CD4+. M, representative plots for flow analysis of MACS enriched peripheral NRP-V7 tetramer+ cells shown in Figure 4D. Analysis is gated on single cells (SSC-A vs SSC-W), B220−Gr1−7-AAD-CD3+CD4−CD8+. 

Analysis is gated on single cells (SSC-A vs SSC-W), B220−Gr1−7-AAD-CD3+CD4−CD8+.