Loss of parity between IL-2 and IL-21 in the NOD Idd3 locus

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IL-2 and IL-21 are two cytokines with great potential to affect autoimmune infiltration of nonlymphoid tissue, and are contained within the strongest non-MHC-linked locus for type 1 diabetes (T1D) susceptibility on the nonobese diabetic (NOD) mouse (Idd3). IL-21 is necessary for the development of diabetes in the NOD mouse, but a number of important studies argue that decreased expression of IL-2 explains Idd3. In this study, we demonstrate that the amount of IL-21, but not IL-2, correlated with T1D incidence. During our analyses of the IL-2/IL-21 interval, we found that mice segregate into one of two distinct expression profiles. In the first group, which includes the C57BL/6 strain, both *Il2* and *Il21* were expressed at low levels. In the other group, which includes the NOD strain, *Il2* and *Il21* were both highly expressed. However, because NOD IL-2 mRNA was relatively unstable, IL-2 production was remarkably similar between strains. The increased production of IL-21 in NOD mice was found to result from two single nucleotide polymorphisms within the distal promoter region that conferred increased binding affinity for the transcription factor Sp1. Our findings indicate that a loss of locus parity after decreased IL-2 mRNA stability ensures that the high-expressing IL-21 allele persists in nature and provides a basis for autoimmunity.

type 1 diabetes | cytokines | transcription factor | allele | promoter

The strongest non-MHC linked locus for type 1 diabetes (T1D) susceptibility in the nonobese diabetic (NOD) mouse (Idd3) encodes two cytokines, *Il2* and *Il21*, which have remained linked throughout evolution. Both IL-2 and IL-21 are essential for various immune functions and can collaborate to drive immune responses. In contrast, they are also known to support the generation of lymphocyte subsets with opposing actions. This complexity is illustrated by the finding that a homozygous deficiency in *Il21r* prevents the onset of T1D in the NOD mouse, whereas a loss of one allele of *Il2* increases disease incidence (15, 23–25). *IL2* and *Il21* are adjacent genes on chromosome 3 in mice, and the analogous locus exists on chromosome 4 in humans. Both IL-2 and IL-21 are known to impact on the disease process, but a genetic lesion that would implicate either gene in the Idd3 effect has not yet been identified (1, 2). Genetic linkage studies support an association of the *IL2*/*IL21* region on human chromosome 4q27 with T1D (3), and this finding has been supported by a recent study in both T1D and RA patients (4).

A decade ago, and before IL-21 was identified, *IL-2* was recognized to be the putative susceptibility gene for Idd3 (1). However, it remained unclear whether IL-2 promoted or inhibited diabetes development. Previous studies had indicated a role for IL-2 in facilitating diabetes development, demonstrating that transgenic expression of IL-2 in the pancreatic islets of NOD mice exacerbated diabetes (5, 6), and that destruction of T cells expressing IL-2R with a diphtheria toxin-related IL-2 fusion protein (DAB486-IL-2) could prevent diabetes (7, 8). In contrast, treatment of NOD mice with the IL-2 mAb (S4B6) facilitated the onset of T1D (9). However, what was not known at that time was that the IL-2 mAb S4B6 does not neutralize IL-2 in vivo as it does in vitro, but instead, potentiates the effect of IL-2 (10). Be that as it may, the observation that a genetic deficiency in IL-2 caused autoimmune disease, as well as the dependency of T regulatory cells (known to suppress autoimmune diabetes) on IL-2, lead to the view that reduced levels of IL-2 in NOD mice might explain the Idd3 locus effect on T1D (11–14). Recent studies in support of this notion demonstrated a relative IL-2 deficiency in NOD mice that correlated with a deficiency in IL-2-dependent regulatory T cells (15). However, other studies have reported that Tregs in the NOD mouse were normal in number and function (16–18).

IL-21 was more recently shown to sit 98 Kb apart from *IL-2* on Idd3, and as such, became a positional candidate for Idd3. IL-21 is produced by activated $CD4^+$ T cells and NK T cells, and can deliver a costimulatory signal to lymphocytes that affects their proliferation, differentiation, and survival in response to antigen (19–21). Consistent with its actions on lymphocyte populations, IL-21 has been shown to have an essential role in an array of conditions involving the destruction of self-tissues (20, 22–24). We have previously shown that NOD mice exhibited elevated levels of IL-21 (26), and a number of recent studies of NOD mice made genetically deficient in the IL-21R demonstrate that IL-21 is necessary for the development of T1D (23–25).

Results

Increased IL-21 in NOD Mice Is Controlled by the Idd3 Locus. Central to this study is our observation that NOD lymphocytes expressed more IL-21 than congenic $NOD^{B6.Idd3}$ mice that carried the B6 IL-21 allele (26). Congenic $NOD^{B6.Idd3}$ mice are NOD mice that carry the Idd3 T1D susceptibility locus encompassing 780 Kb of chromosome 3 from C57BL/6 mice. The incidence of diabetes in $NOD^{B6.Idd3}$ mice is reduced to 20% compared with 80% of NOD mice (27). We extended our analyses to the kinetics of IL-21 mRNA production in purified $CD4^+$ T cells. IL-21 mRNA was transcribed from both groups with the same kinetics, but NOD $CD4^+$ T cells expressed more IL-21 mRNA than $NOD^{B6.Idd3}$ mice (Fig. 1*A*). To confirm the increase in IL-21 at a protein level, we compared IL-21 produced from naive (CD44lo) $CD4^+$

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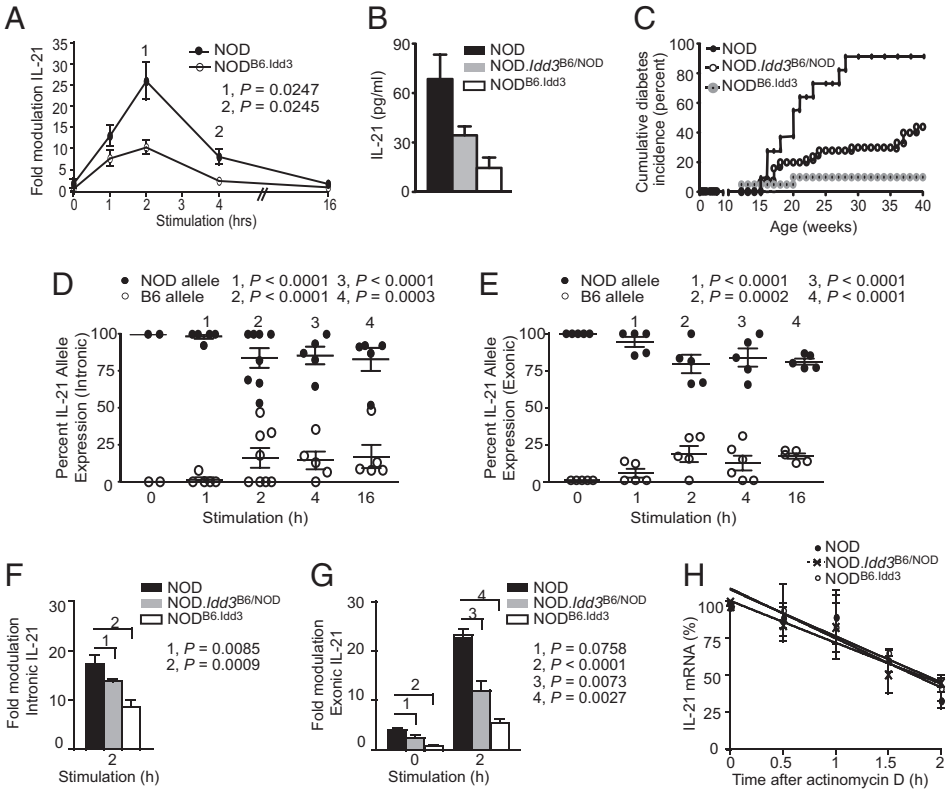

Fig. 1. CD4$^+$ T cells from NOD mice have increased production of IL-21. (*A*) Sorted, activated CD4$^+$ T cells stimulated with PMA and Ionomycin and IL-21 mRNA expression measured by real-time PCR at times shown. Values are presented as fold modulation (relative units) relative to unstimulated NOD$^{B6.Idd3}$ cells. Data are presented as mean ± SEM, n = 6–9 mice per group, three experiments. (*B*) Splenocytes from NOD, NOD.*Idd3*$^{NOD/B6}$, and NOD$^{B6.Idd3}$ mice stimulated for 2 days with CD3 and CD28 mAb. IL-21 measured by ELISA and data presented as mean ± SEM, n = 6 mice per group, two experiments. (*C*) Cumulative incidence of diabetes in NOD, n = 15; NOD.*Idd3*$^{NOD/B6}$, n = 18; and NOD$^{B6.Idd3}$ mice, n = 20. (*D*) Intronic IL-21 (premRNA) measured by allele specific pyrosequencing assay in CD3 and CD28 mAb stimulated splenocytes from NOD.*Idd3*$^{NOD/B6}$ mice, n = 18–28 mice per group, three experiments. (*E*) Exonic IL-21 mRNA measured by pyrosequencing in CD3 and CD28 mAb stimulated NOD.*Idd3*$^{NOD/B6}$ splenocytes. (*F*) Intronic and (*G*) exonic IL-21 mRNA expression measured in NOD, NOD.*Idd3*$^{NOD/B6}$, and NOD$^{B6.Idd3}$ splenocytes stimulated with CD3 and CD28 mAb at times shown, n = 15 mice per group, four experiments. (*H*) Decay of exonic IL-21 mRNA from stimulated NOD, NOD.*Idd3*$^{NOD/B6}$, and NOD$^{B6.Idd3}$ splenocytes treated with actinomycin D for the times indicated. Data shown as mean ± SEM where n = 6 per group, two experiments.

T cells from NOD, NOD$^{B6.Idd3}$ mice, and an F1 cross of NOD × NOD$^{B6.Idd3}$ mice, producing NOD mice heterozygous at the Idd3 locus (NOD.Idd3$^{B6/NOD}$), after activation in vitro. A significantly greater amount of IL-21 protein (3-fold) was produced from naive CD4$^+$ T cells from NOD mice compared with NOD$^{B6.Idd3}$ mice and that NOD.Idd3$^{B6/NOD}$ mice produced an intermediate amount of IL-21 (Fig. 1*B*). Correlating with intermediate IL-21 levels, the incidence of diabetes was 40% in F1 mice, compared with 90% of NOD and 10% of Idd3 mice (Fig. 1*C*).

Increased Expression of the NOD IL-21 Allele. The increased levels of IL-21 mRNA and protein in NOD CD4$^+$ T cells prompted us to examine the relative activities of the NOD and B6 IL-21 alleles. We used the progeny of an F1 cross of NOD × NOD$^{B6.Idd3}$ mice (NOD.Idd3$^{B6/NOD}$) for the analyses, which offered an important internal control by allowing us to determine the abundance of each IL-21 allele in an equivalent genetic background. We used primers that span a polymorphism in intron 2 of the IL-21 gene to distinguish between the NOD IL-21 allele and B6 IL-21 allele in NOD.Idd3$^{B6/NOD}$ mice heterozygous at the Idd3 locus after stimulation in vitro with CD3 and CD28 mAbs. Our analyses revealed that expression of the NOD IL-21 allelic variant dominated the expression of the B6 IL-21 allele, resulting in an abundance of both premRNA (intronic) and total mRNA (exonic) in NOD splenocytes (Fig. 1 *D* and *E*, respectively), pooled lymph nodes (LNs), lymphocytes purified from the pancreas (Fig. S1), including both purified activated and naive CD4$^+$ T cells (Fig. S1).

A complementary analysis of IL-21 levels by real-time PCR confirmed the greater activity of the NOD IL-21 allele and showed that the expression of intronic IL-21 mRNA (Fig. 1*F*) and exonic IL-21 mRNA (Fig. 1*G*) from the F1 hybrid mice was greater than B6 and less than NOD. This result indicates that there was no apparent dominant effect between trans-regulating loci from one background and allelic loci from the other background. The increased expression of the NOD IL-21 allele did not reflect a difference in mRNA stability (Fig. 1*H*; Fig. S1), and therefore, was likely to reflect regulation of IL-21.

Promoter Polymorphisms Increase IL-21 Levels Through Sp1 Binding. The increased IL-21 levels in NOD mice might have reflected sequence variation in regulatory components of the NOD and B6 IL-21 alleles. A comparative analysis of a generous 2-Kb region of the NOD and B6 IL-21 promoters identified 30 sequence single nucleotide polymorphisms (SNPs), 13 of which were located at putative transcription factor binding sites in the NOD allele (Fig. S2 and Table S1). The IL-21 proximal promoters from both NOD and B6 mice contain nuclear factor of activated T cells (NFAT) binding sites that are essential for promoter activity (28, 29). Therefore, nested promoter constructs were designed that encompassed the IL-21 proximal promoter and sequential polymorphic transcription factor bind-

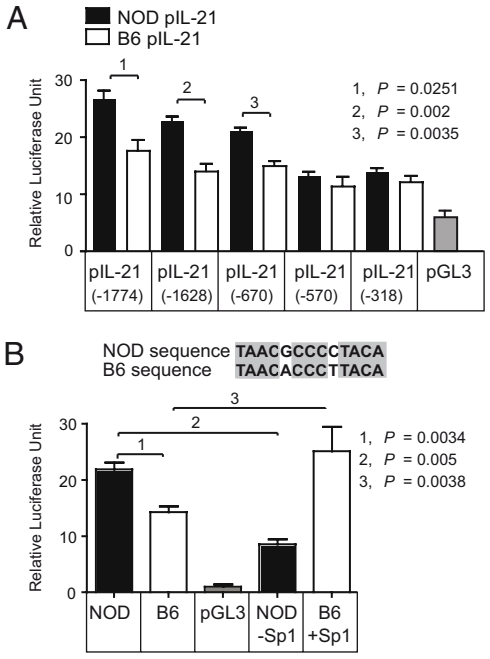

Fig. 2. The NOD IL-21 promoter exhibits increased transcriptional activity. (*A*) IL-21 promoter activity measured in primary CD4$^+$ T cells transfected with IL-21 promoter/luciferase reporter gene constructs, after 8-h stimulation with PMA and ionomycin. Data are presented as the mean ± SEM. (*B*) Site directed mutagenesis (SDM) was used to convert the Sp1 site in the NOD promoter (pIL-21 −1774) to the equivalent B6 site (NOD-Sp1) or create a NOD Sp1 site in the B6 promoter (B6+Sp1) in EL4 cells. Results are presented as the mean ± SEM, n = 3–4, for each luciferase assay.

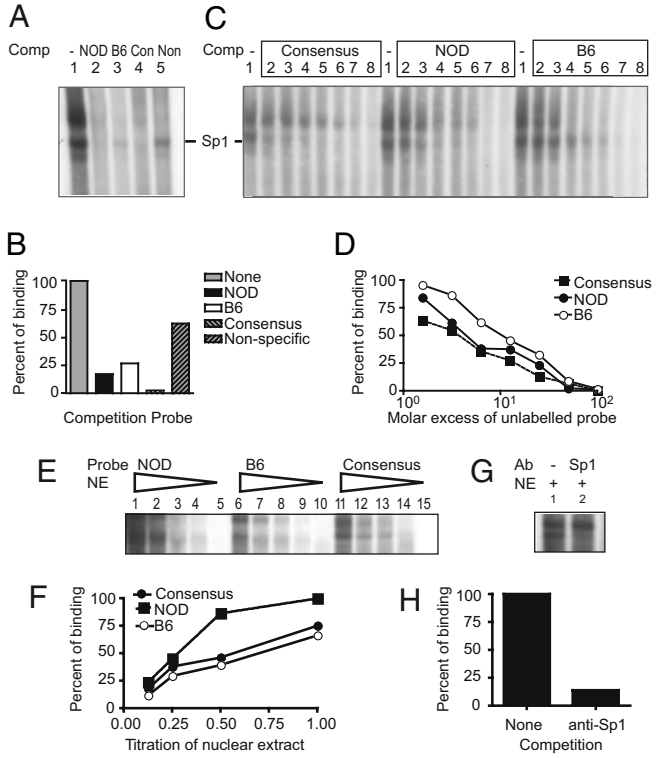

Fig. 3. The NOD IL-21 promoter exhibits increased Sp1 binding. (*A*) Electromobility shift assay showing Sp1 binding complex on the IL-21 promoter. Stimulated EL4 nuclear extracts (NE) were incubated with ^{32}P labeled probe specific for the NOD Sp1 binding site, in the absence of competitor (lane 1), or in competition with unlabeled NOD (lane 2), B6 (lane 3), consensus (Con) (lane 4), or nonspecific (Non) (lane 5) probes. (*B*) The level of inhibition for each Sp1 probe is shown relative to the total binding (100%). Data are representative of two experiments. (*C*) ^{32}P labeled consensus Sp1 probe was titrated 1:2 (right to left) against 100-fold molar excess unlabeled consensus, NOD, or B6 probe. (*D*) Intensity of complexes relative to total binding (100%). Data are representative of two experiments. (*E*) EL4 NE was titrated over radio labeled probes derived from NOD, B6, or consensus sequence as indicated. (*F*) The intensity of complexes is shown relative to NOD Sp1 probe in the presence of excess EL4 NE. Data are representative of two experiments. (*G*) Binding of the NOD probe is lost when NE is incubated with Sp1 mAb (Ab) (lane 2). (*H*) Binding with Sp1 mAb is shown relative to total binding. Data are representative of two experiments.

ing sites that spanned the entire 2-Kb promoter region. These studies revealed a significant decrease in transcription of the reporter gene in the NOD promoter segments that excluded an Sp1-binding site containing two polymorphisms between −591 and −582 (Fig. 2*A*).

The transcription factor Sp1 is a zinc finger protein that boosts the expression of a number of genes by binding to a specific regulatory DNA sequence (consensus Sp1 site) located in the promoter region (30). To test whether an Sp1-like protein positively regulated transcription of the NOD IL-21 allele, we used site-directed mutagenesis to change the two SNPs in the NOD IL-21 promoter Sp1 site and convert it to a B6 site. This conversion effectively reduced the NOD IL-21 promoter activity to the levels of the B6 IL-21 promoter (Fig. 2*B*). The vice versa conversion of the B6 site to a NOD Sp1 site substantially increased B6 IL-21 promoter strength (Fig. 2*B*). Analyses of the binding of Sp1 from activated T cell nuclear extracts to the NOD and B6 IL-21 promoter were consistent with the polymorphic NOD Sp1-binding site conferring an increased affinity for Sp1 (Fig. 3). An excess of both the unlabelled NOD Sp1 and control consensus Sp1 binding probes effectively competed with a radio-labeled NOD Sp1 probe (Fig. 3 *A* and *B*) and a radio-labeled NOD consensus Sp1 probe for Sp1 binding (Fig. 3 *C* and *D*; Fig. S3). In contrast, the B6 probe was unable to compete with either the NOD or consensus probe (Fig. 3 *C* and *D*), suggesting a reduced affinity for Sp1. Similarly, the B6 probe exhibited a comparatively poor ability to bind Sp1 (Fig. 3 *E* and *F*). Last, an antibody to Sp1 blocked binding of the NOD Sp1 probe, which confirmed the presence of a specific Sp1 complex in the nuclear extract (Fig. 3 *G* and *H*). Together, these studies demonstrated an important role for Sp1 in improving the transcription efficiency of the NOD IL-21 promoter.

NOD IL-2 Allele Is Highly Expressed, but the mRNA Is Less Stable. We performed parallel analyses of the NOD and B6 IL-2 alleles in mice heterozygous at the Idd3 locus (NOD.Idd3$^{B6/NOD}$ mice), and observed that intronic NOD IL-2 mRNA allele was also transcribed with high efficiency relative to the B6 IL-2 allele, resulting in a significantly greater amount of the NOD IL-2 allele from in vitro activated splenocytes (Fig. 4*A*), LN suspensions, and lymphocytes extracted from the pancreas (Fig. S4). However, when we analyzed exonic (total) IL-2 mRNA, we observed equivalent amounts of the NOD IL-2 and B6 IL-2 alleles (Fig. 4*B*; Fig. S4). Complementary analyses of IL-2 mRNA expression from NOD, NOD$^{B6.Idd3}$, and NOD.Idd3$^{B6/NOD}$ mice demonstrated equivalent levels of IL-2 mRNA in these mice, and also showed incongruous expression of the intronic and exonic NOD IL-2 mRNAs, respectively (Fig. 4 *C* and *D*). Analysis of IL-2 mRNA decay in stimulated splenocytes after inhibition of transcription by actinomycin D indicated that the disparity between the intronic and total IL-2 mRNA in NOD mice could be explained by a decreased stability of NOD IL-2 mRNA (half-life of 72 min) compared with B6 IL-2 mRNA (half-life of 85 min) (Fig. 4*E*; Fig. S4).

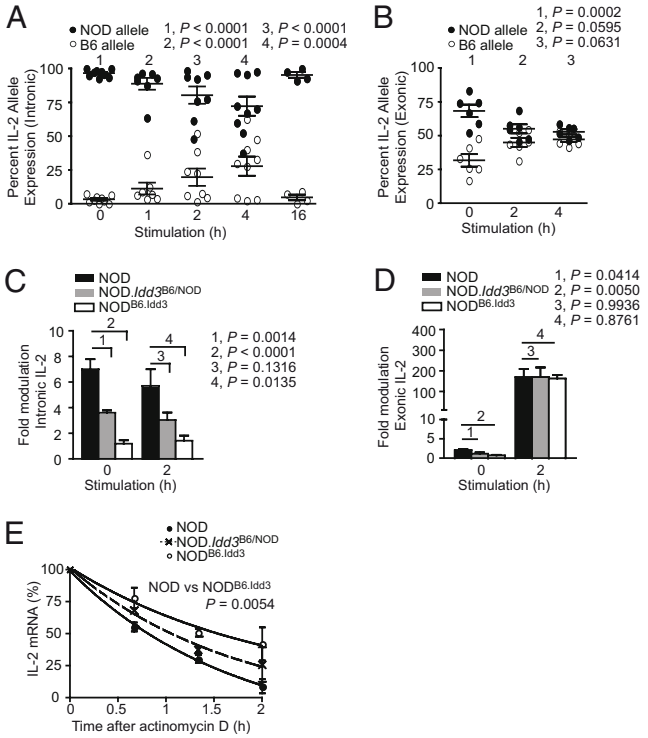

Fig. 4. Decreased stability of NOD mRNA. (*A*) Intronic IL-2 (premRNA) and (*B*) exonic IL-2 mRNA measured by allele specific pyrosequencing assay from NOD.*Idd3*$^{NOD/B6}$ splenocytes stimulated with CD3 and CD28 mAb, $n = 15$ mice per group, four separate experiments. (*C*) Intronic and (*D*) exonic IL-2 mRNA expression measured by real-time PCR from NOD, NOD.*Idd3*$^{NOD/B6}$, and NOD$^{B6.Idd3}$ splenocytes stimulated as above, $n = 15$ mice per group, four experiments. (*E*) Decay of exonic IL-2 mRNA from stimulated NOD, NOD.*Idd3*$^{NOD/B6}$, and NOD$^{B6.Idd3}$ splenocytes treated with actinomycin D for the times indicated. Data shown are mean values ± SEM where $n = 6$ per group, from two experiments.

To assess functional levels of IL-2 protein in NOD versus NOD$^{B6.Idd3}$ mice, we took advantage of a system described recently, where IL-2 mAb (S4B6) was administered to mice to form a complex with bioavailable IL-2 protein that preferentially expands memory phenotype CD8+ T cells (10). This method demonstrated a linear relationship between the amount of IL-2 administered and the proliferation of 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE)-labeled CD8+ T cells (Fig. S5), and could detect a 50% reduction in proliferation of memory phenotype CD8+ T cells in IL-2$^{+/-}$ compared with WT mice (10). IL-2:IL-2mAb-responsive CD8+ T cells expanded to the same extent in NOD and NOD$^{B6.Idd3}$ mice (Fig. S5). Also, transferred CFSE-labeled CD8+ T cells from NOD.Idd3$^{B6/NOD}$ (F1) mice proliferated to an equivalent extent in NOD and NOD$^{B6.Idd3}$ mice treated with anti-IL-2 mAb, indicating equivalent amounts of IL-2 in NOD and NOD$^{B6.Idd3}$ mice (Fig. 5 *A* and *B*). A direct comparison of IL-2 production from T cells from the pancreas by intracellular immunostaining and FACs analyses demonstrated that there were more IL-2-producing T cells infiltrating the pancreas of NOD mice (Fig. S5). However, the CD4$^+$ T cells (Fig. 5 *C* and *D*) and CD8+ T cells (Fig. 5*E*; Fig. S5) from the spleen and pancreata of NOD and NOD$^{B6.Idd3}$ mice produced equivalent amounts of IL-2. In accordance with these findings, IL-2 was detected equally in T cells from NOD and NOD$^{B6.Idd3}$ mice by Western blotting (Fig. 5*F*).

Expression Profiles for IL-2 and IL-21 form Two Distinct Groups. To further probe the relationship between IL-2 and IL-21 expression, we similarly analyzed five additional mouse strains. We used allele-specific pyrosequencing to analyze the progenies of F1 crosses of CBA × C57BL/6, DBA × C57BL/6, DBA × BALB.c, C3HeJ × C57BL/6, 129 × C57BL/6, and BALB/c × C57BL/6 mice to determine the relative abundance of the IL-2 and IL-21 alleles. Our analyses identified two distinct expression patterns for IL-2 and IL-21. In one, found in the C57BL/6 and BALB/c strains, IL-21 and IL-2 (Fig. S6) were both expressed at moderate levels. In the other, found in the DBA, CBA, C3HeJ strains, and 129 strain, which contains an identical Sp1 site to NOD, both IL-21 and pre (intronic) IL-2 mRNA were highly expressed (Fig. S6). IL-21 mRNA decay was similar between strains. However, in the latter group, the convergence of exonic IL-2mRNA levels was explained by increased decay of IL-2 mRNA (Fig. S6). Analyses of the IL-21 promoter regions of these strains indicated that the NOD-like group all contained the two SNPs that created the high-affinity sp1-binding site (Table S2). Together, these findings indicated that the parity between the expression of the linked IL-2 and IL-21 alleles was lost through greater instability of NOD IL-2 mRNA, resulting in a disproportionately high expression of IL-21 (Fig. S7).

Discussion

The profound impact of the Idd3 locus on the susceptibility to insulitis and T1D is consistent with the notion that multiple genes on the locus contribute to the T1D susceptibility. IL-21 is necessary for the development of diabetes in the NOD mouse (23–25), but a number of important studies argue that decreased expression of IL-2 explains Idd3 (14, 15, 31). Our findings resonate with a critical role for IL-21 in T1D pathogenesis, but do not formally discount a role for IL-2. We revealed that the amplification of IL-21 expression in the NOD mouse was, in part, due to two SNPs that improved binding of the transcription factor Sp1 to the IL-21 promoter. Sp1 is increased in T cells on stimulation through the TCR (32), and has been demonstrated to drive TCR-induced IL-21R expression on T cells (32), indicating that Sp1 could modulate both the IL-21 protein and its receptor. However, additional effects from coordinate regulation of promoter activity (suggested by the equally strong initiation of transcription of IL-2) warrant further examination.

One scenario that could reconcile a role for both IL-2 and IL-21 in T1D is that a defect in T regulatory cells (33, 34) caused by reduced IL-2 levels might foster the IL-21-driven expansion of diabetogenic T cells. Indeed, studies demonstrating that depletion of IL-2-dependent T regulatory cells improved the efficacy of IL-21 in cancer immunotherapy (35), and IL-21 suppressed FoxP3+ T regulatory cells (36) argue that these two cytokines could influence each other. In accordance with a greater infiltration of mononuclear cells, there were a greater number of IL-2 producing T cells in the pancreata of NOD mice compared with NOD$^{B6.Idd3}$. However, we did not observe any difference in IL-2 mRNA or IL-2 protein production from NOD and NOD$^{B6.Idd3}$ T cells. In support of this finding, a number of recent studies have demonstrated no deficit of Tregs in the primary or secondary lymphoid organs of NOD mice (16–18).

Our findings contrast with a previous study demonstrating an ≈50% reduction in the expression of the NOD IL-2 allele compared with the B6 IL-2 allele (15). The reasons why our results differ remain unknown, but may reflect different experimental approaches for IL-2 measurements. For example, the previous study measured IL-2 protein by ELISA from cultured TCR transgenic CD8+ T cells (15), whereas we analyzed IL-2 protein in NOD and NOD$^{B6.Idd3}$ mice in vivo with a method that utilizes IL-2 mAb (S4B6) to complex endogenous IL-2 to quantitatively measure levels of IL-2 protein (10). Our analyses of IL-2 mRNA after timed stimulation of lymphocytes in vitro contrasted with the analyses of cells from mice that had been injected with CD3 mAb (15). Stimulation of T cells in vitro is not

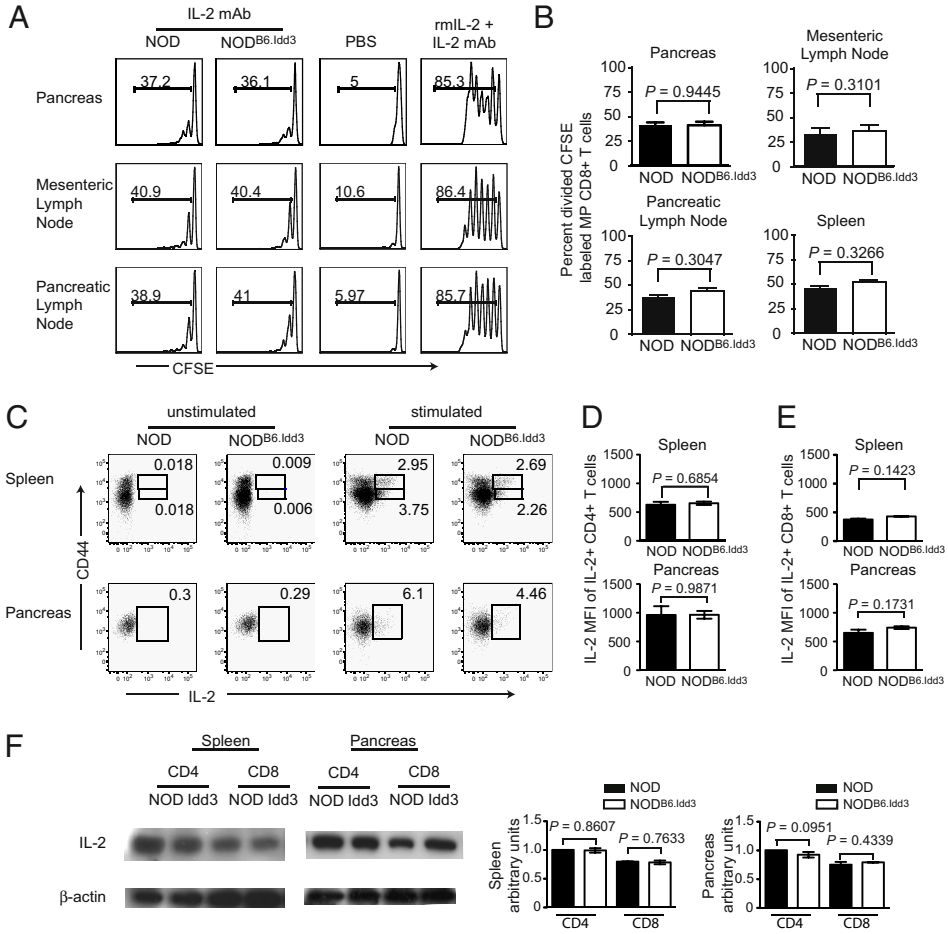

Fig. 5. Equivalent amounts of IL-2 in NOD and NOD$^{B6.Idd3}$ mice. (*A*) Representative histograms showing CFSE dilution of transferred NOD.*Idd3*$^{NOD/B6}$ (Idd3 F1) CD122+ CD44^{hi} MP CD8+ T cells in NOD and NOD$^{B6.Idd3}$ hosts after daily i.p. injections of S4B6, PBS, or S4B6+rmIL-2 examined for the markers shown on day 7, (*B*) shown as percentage divided CD122+ CD44^{hi} MP CD8+ T cells where $n = 6$, from two experiments. (*C*) Representative dot plots from flow cytometric analyses of IL-2 in NOD and NOD$^{B6.Idd3}$ CD4$^+$ T cells directly ex vivo (unstimulated) and after 5-h stimulation of splenocytes and islet-extracted lymphocytes with PMA and Ionomycin. Mean fluorescence intensity (MFI) of IL-2 in CD4$^+$ T cells (*D*) and CD8+ T cells (*E*) from the spleen and pancreas of NOD and NOD$^{B6.Idd3}$ mice, $n = 8$, from three experiments. (*F*) IL-2 detected by Western blot analysis in total lysates of CD44hi CD4$^+$ and CD8+ T cells with band intensity quantified in arbitrary relative units, representative of three experiments.

fully compatible with what happens in vivo, but it offers the advantages of analyzing expression kinetics. However, additional mechanisms that may regulate and even coordinate IL-2 and IL-21 gene expression, including regulation by effects from microRNA, are likely to play a role.

Possessing a gene that produces a large amount of IL-21 was important for T1D development, but increased production of IL-21 alone was not sufficient in the absence of other susceptibility alleles. Also, this allele was not specific to the NOD mouse. In fact, DBA, CBA, and C3HeJ all joined the NOD in expressing the high IL-21 allele and the less stable IL-2 mRNA. It is notable that we observed only two expression profiles for IL-2 and IL-21, and we have yet to observe a strain that has high expression levels of both IL-2 and IL-21. This finding suggested that this combination (or high expression of IL-2 *per se*) was not common in inbred mouse strains. The decreased stability of NOD IL-2 mRNA indicated that posttranscriptional modifications exist in the NOD IL-2 allele, which may ensure the maintenance of a high expressing IL-21 allele that contributes to the development of autoimmune disease.

Methods

Mice. NOD Ltj and C7BL/6 mice were obtained from ARC. The congenic-C57BL/6(R450).NOD (NOD$^{B6.Idd3}$) mice were purchased from Taconic Farms. The *Il21*$^{-/-}$ mice (Lexicon) were C57BL/6 N5. Animals were housed under specific-pathogen-free (SPF) conditions and handled in accordance with the Garvan Institute of Medical Research and St. Vincent's Hospital, Darlinghurst, NSW, Austalia, Animal Ethnics Committee (AEC). Blood glucose values (BGV) were determined using Accu-chek Advantage blood glucose strips (Roche).

Quantitative (q)RT-PCR. The relative amounts of cDNAs were determined in triplicate by qRT-PCR analysis using the ABI Prism 7700 Sequence Detection System (Applied Biosystems), positive samples were located in linear range of standard curve. Real-time PCR primers are shown in Table S3 or previously described (15). Fold modulation was calculated by employing a comparative CT method [relative abundance of genes = 2({Delta}Ct), where {Delta}Ct is the difference between the Ct of target and the arithmetic mean of Cts of GAPDH], with an additional correction for CD4$^+$ T cell percentage/sample.

Pyrosequencing. Pyrosequencing was performed on cDNA synthesized from the DNase treated RNA. Assays were performed according to the manufacturer's instructions on the PSQ96 system (Biotage). Oligonucleotides used to quantitate the NOD and B6 intronic IL-21 alleles in NOD.*Idd3*.$^{B6/NOD}$ mice are shown in Table S3. Oligonucleotide primers used to quantitate NOD and B6 intronic IL-2 alleles were described previously (15).

In Vitro Cell Stimulation. Naive (CD44lo) and memory (CD44hi) CD4$^+$ T cells were sorted (FACSAria; BD Biosciences) from NOD and NOD$^{B6.Idd3}$ spleen, LNs, and thymus, and stimulated as shown in *SI Methods*.

Administration of IL-2 Antibodies in Vivo. Macs sorted NOD.*Idd3*$^{NOD/B6}$ CD8+ T cells (>90% pure) were CFSE labeled and 8×10^6 injected i.v., followed by daily i.p. injections of PBS, S4B6 IL-2 mAb (50 μg), or rmIL-2 (1.5 μg) (Peprotech) plus IL-2 mAb (50 μg) as previously described (10). Donor and host cells from spleen and LNs were examined on day 7. S4B6 was purified in house from the S4B6.1 hybridoma.

SNP Promoter Identification and Luciferase Assay. The extended IL-21 promoter regions (from −2182 to +125), generated by PCR using KOD polymerase (Novagen) from NOD and C57BL/6 genomic DNA, and nested promoter constructs were cloned as shown in *SI Methods*. The promoter inserts were sequenced, aligned (Fig. S2), and analyzed using the TRANSFAC database (http://www.cbil.upenn.edu/tess/).

Electromobility Shift Assay. The sequences of the oligonucleotides used to investigate the putative Sp1 binding site in the NOD IL-21 promoter are shown in Table S3 and *SI Methods*.

Intracellular Staining of IL-2. Splenocytes and pancreas isolated lymphocytes (as shown in *SI Methods*) from NOD and NOD$^{B6.Idd3}$ mice were cultured with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 500 ng/mL Ionomycin for 5 h in the presence of Golgi Plug. Cells were stained with CD4 and CD44 mAb, fixed and permeabilized (ebioscience) and stained intracellular with IL-2 mAb (BD Bioscience).

Western Blotting. SDS/PAGE was performed on total cell lysates of CD4$^+$ and CD8+ T cells sorted from NOD and NOD$^{B6.Idd3}$ pancreas isolated lymphocytes, and blotted for mouse IL-2 (clone JES6–1A12).

Statistical Analysis. *P* values between datasets were determined by two-tailed Student's *t* test assuming equal variance.

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