



## Pre-existing autoimmunity determines type 1 diabetes outcome after Flt3-ligand treatment

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### ABSTRACT

Redirection of immune responses by manipulation of antigen-presenting cells is an emerging strategy for immunosuppressive treatment of autoimmune diseases. In vivo expansion of dendritic cells (DC) by Fms-like tyrosine kinase-3 (Flt3)-Ligand (FL) treatment was shown to delay diabetes onset in the NOD model of autoimmune diabetes. However, we show here that Flt3 stimulation actually accelerates autoimmunity when autoreactive CD8 T cells are detectable in blood prior to treatment. With autoreactive CD8 cells present, the capacity of FL to expand DCs and induce Treg remained intact, but both numbers and the functional response of islet-specific CD8s were boosted. Also, the inhibitory receptor PD-1 on (autoreactive) CD8 T cells and its ligand PD-L1 on Treg were no longer upregulated. These data highlight the need to pre-screen for T cell autoreactivity prior to generalized DC expansion and illustrate how accelerated disease can occur when the intended initiation of regulatory mechanisms is impaired later in diabetogenesis.

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### 1. Introduction

Type 1 diabetes mellitus (T1D), also called juvenile diabetes, is a chronic autoimmune disorder that precipitates in genetically susceptible individuals in conjunction with environmental factors [1]. The body's own immune system destroys or damages the  $\beta$ -cells in the pancreatic islets of Langerhans sufficiently to reduce and eventually eliminate insulin production, which subsequently leads to hyperglycemia. Many of the current successes in preclinical treatment of T1D are achieved in preventive but not therapeutic settings [2]. On the other hand, many clinical trials assess the efficacy of treatments in a therapeutic setting in patients with recent-onset T1D. Consequently, there can be a strong disconnect between preclinical studies and clinical investigations in terms of the time of intervention. This likely explains why some treatment strategies that are successful in NOD mice do not show a notable effect in human disease [2]. Here, we address the late stage efficacy of a novel treatment that had shown preclinical preventive success, namely the restoration of dendritic cell (DC) imbalance in NOD mice by administration of Fms-like tyrosine kinase 3 (Flt3) ligand (FL).

Phenotypic, maturation and functional abnormalities of DCs have been suggested to contribute to autoimmunity in human T1D

and NOD mice [3–5]. As such, blood DC numbers are reduced in T1D children [6] and the plasmacytoid DC frequency is lower in T1D patients [7]. The NOD mouse displays reduced overall DC numbers [8] plus a DC imbalance with too little CD8 $\alpha^+$  DCs [8,9], and too many CD8 $^-$ CD11c $^+$  DCs [10] or CD11b $^+$ CD11c $^+$  myeloid DCs [11]. Moreover, it has been suggested that impaired Treg activation by NOD DCs allows the autoreactive T cells to cause  $\beta$ -cell destruction [12]. It is therefore conceivable that overcoming this DC defect could correct the autoimmunity in the NOD. For example, transferring high numbers of NOD pancreatic LN-derived DC prevents diabetes onset [13]. In vivo expansion of DC by FL treatment during early prodromal stages was shown to delay diabetes onset in the NOD model [8,14]. FL is a cytokine growth factor known to mobilize hematopoietic precursors into peripheral blood [15,16]. Flt3 receptor is absent on all major cell lineages (B, T, NK, granulocytes, monocytes/macrophages) except DCs [17]. CD8 $\alpha^-$  and CD8 $\alpha^+$  DCs express more Flt3 than B220 $^+$  pDCs [17,18] but FL nevertheless expands all DC populations both in vivo [18] and in vitro [18,19]. FL treatment also induces regulatory T cells [14,20], suggesting that FL prevents diabetes onset by increasing the potential to suppress autoimmune responses.

In an effort to investigate the clinical relevance and potential adverse effects of such a generalized DC expansion in the treatment of autoimmune diabetes, we conducted a precise analysis of therapeutic success in correlation with the disease stage and the underlying mechanisms. We found that FL treatment is not beneficial at advanced stages of T1D development that correspond with

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the clinical diagnosis of type 1 diabetes: at that time, FL enhanced pre-existing CD8 autoreactivity thereby accelerating diabetes onset, rather than protecting from it.

## 2. Methods

### 2.1. Mice and diabetes monitoring

Female NOD Lt/J (NOD) mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and housed in micro-isolator cages under specific pathogen-free conditions at the La Jolla Institute for Allergy and Immunology. All animal studies were performed according to institutional and National Institutes of Health guidelines for animal use and care. Blood glucose (BG) levels were monitored weekly from the tail vein using OneTouch Ultra (Life-Scan Inc.). Diabetes incidence was dated on the first of two consecutive BG values >200 mg/dl.

### 2.2. Flt3-ligand treatment

Human Flt3-Ligand (huFL) was received from Amgen (Thousand Oaks, CA, USA). FL treatment regimen was a 10-day course of 10 µg human FL daily given intraperitoneally in 50 µl carrier solution. Control group was given carrier alone (25 mM Tris-HCl, 4% mannitol, 1% sucrose).

### 2.3. Flow cytometry

Fluorochrome-labeled monoclonal antibodies were obtained from BD Biosciences, eBioscience, BioLegend, or Caltag. The H-2K<sup>d</sup> tetramer refolded with the NRP-V7 peptide (KYNKANVFL) was obtained from Rusung Tan (Vancouver, Canada) [21]. Tetramer staining was performed at RT for 1 h. For DC subset analysis, single-cell suspensions were obtained by collagenase D digestion and incubated for 20 min at 6–8 °C with the following panel of mAbs: anti-CD3ε (145-2C11), CD11c (HL3), B220 (RA3-6B2), CD24 (M1/69), CD172 (P84), CD8α (5H10), MHC Class II (39-10-8 for NOD). For T cell subsets: CD3ε (145-2C11), CD4 (RM-4.5), CD8α (5H10), CD25 (PC61), FoxP3 (FJK-16s), CD19 (1D3), PD-1 (RMP1-30) and biotinylated PD-L1 (MIH5) were both from eBioscience. FoxP3 staining was performed using eBioscience reagents. To determine autoreactive CD8 responses by intracellular cytokine staining, cells were restimulated in vitro for 5 h with 1 µg/ml NRP-V7 peptide (KYNKANVFL) in the presence of IL-2 and Brefeldin A. In Fig. 3, in vitro restimulation and ICCS were preceded by tetramer staining. For blood and pancreas, T-cell depleted NOD splenocytes were added to provide better presentation of antigen. Surface staining was fixed in 1% (para) formaldehyde before intracellular staining for IL-17A, IFN-γ and TNF-α in fresh permeabilizing buffer (PBS, 0.5% BSA, 0.1% saponin) for 45 min at 6–8 °C. Stained samples were acquired on LSRII.

### 2.4. Screening for IGRP-specific CD8 T cells

Detection of IGRP-specific CD8 T cells in peripheral blood was done as previously described [22]. Briefly, blood (130 µl) was collected by retro-orbital bleeding and placed in PBS with 2 mM EDTA. Next, erythrocytes were removed by ACK lysis and stained with NRP-V7/H-2K<sup>d</sup> tetramer (1:160) for 1 h at RT, with addition of anti-CD8 and anti-B220 mAbs during the last 10 min without intermittent wash. Flow cytometric analysis was used to determine the frequency of NRP-V7/K<sup>d</sup> positive cells in the CD8<sup>+</sup>CD19<sup>-</sup> population. Mice were assigned to the 'IGRP-CD8 pos' group when this frequency was >0.5% on a single measurement or >0.75% of cumulated measurements over x number of weeks, where x varies depending on the start of treatment. Within the 'IGRP-CD8 pos'

or 'IGRP-CD8 neg' groups, mice were then randomized into control groups or FL treatments. The cumulative frequencies of IGRP-specific CD8s did not differ between treatment groups (Supplementary Fig. 2).

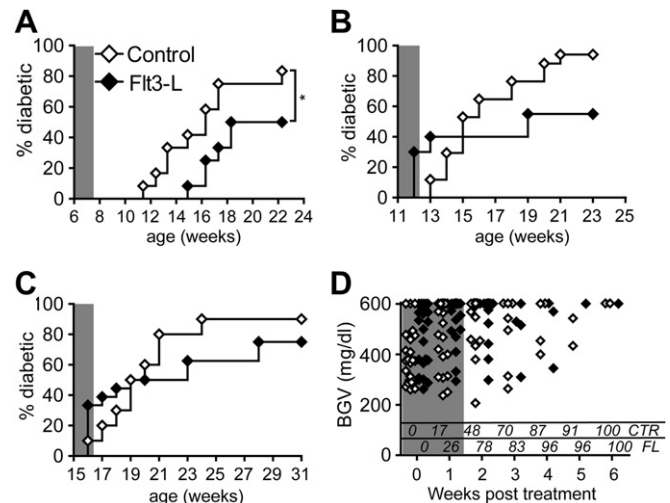
### 2.5. Statistical analysis

For diabetes incidence, significance was calculated by logrank test (Mantel–Cox). For cell subset analysis, non-parametric *t*-test Mann–Whitney was used. Paired *t*-test was used on NRP-V7 responses in blood before versus after FL treatment (Fig. 5b). Student's *t*-test was used for the CD135 expression in Supplementary Fig. 1. Statistical significance is indicated as follows: *p* < 0.05 (\*), *p* < 0.01 (\*\*) and *p* < 0.005 (\*\*\*).

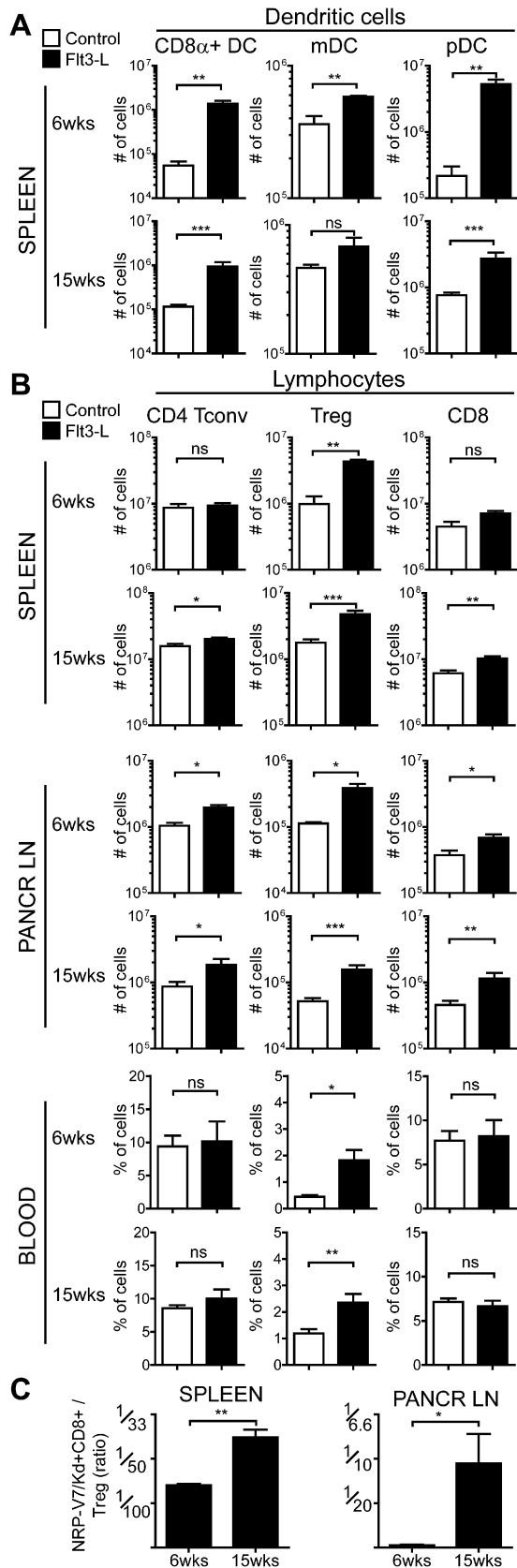
## 3. Results

### 3.1. Late stage FL treatment can accelerate diabetes

We based the design of our current study on the previous findings that FL can delay the onset of T1D in NOD mice when administered early during diabetes development [8,14] but that FL did not protect when administered during later stages [20]. It was possible that the differential outcome of early versus late stage treatment in these studies was simply due to the lower efficacy of murine FL (muFL) as compared to human FL (huFL) used in the early prevention study. Indeed, repeated treatment regimens of muFL are required to provide similarly strong preventive protection as a single regimen of huFL [8]. In addition, it is known that overall DC numbers are increased most effectively with huFL [8], even though muFL enhances CD8α<sup>+</sup> cDC and pDC subtypes more selectively than huFL. We therefore chose to assess the effect of a 10-day regimen of daily intraperitoneal doses of the more potent huFL during different stages of disease. We treated normoglycemic NOD mice starting either at 6 weeks, 11 weeks, 13 weeks (data not shown), or 15 weeks, as well as recent-onset NOD mice. Our results confirmed that early administration of FL protected NOD mice from diabetes



**Fig. 1.** FL treatment selectively accelerates diabetes onset during later stages of T1D development. Female NOD mice were treated i.p. for 10 consecutive days with 10 µg huFL in carrier solution (filled symbols) starting A: at 6 weeks, B: 11 weeks, C: 15 weeks or D: at diabetes onset; control groups received carrier solution alone (open symbols); *n* = 12 or more/group. Blood glucose values were monitored before, during (shaded area) and after treatment. Diabetes onset was dated on the first of 2 consecutive readings >200 mg/dl. Only mice normoglycemic at the start of the treatment are included in the data in panel A, B and C. In panel D, the inset values (in italics) represent the percentage of mice that were moribund or dead at each time-point for control- (top row) or FL-treated (bottom row) mice.



(Fig. 1A). Somewhat different from a study by Cheatem et al. we found that late stage huFL treatment of normoglycemic NOD mice resulted in a dual effect on disease onset: one part of the FL-treated group developed diabetes very rapidly, while the other part of the cohort showed a strong delay in diabetes onset (Fig. 1B,C). We observed this when treatment was started in normoglycemic NOD mice at 11, 13, or 15 weeks of age. This segregation was still valid when blood glucose concentrations of 250 mg/dl instead of 200 mg/dl were used as threshold for diabetes onset (data not shown). Importantly, FL treatment was unable to cure diabetes and we observed a trend towards increased morbidity when administered to hyperglycemic NOD mice (Fig. 1D and data not shown). Of note, diabetic NOD mice that receive no or control treatment never revert to normoglycemia (data not shown). Together, these data indicate that FL treatment is not always protective, but can also aggravate disease. We reasoned that the degree of pre-existing autoimmunity and islet inflammation would determine treatment success or failure.

### 3.2. Similar increase in DC and Treg numbers during late compared to early stage treatment

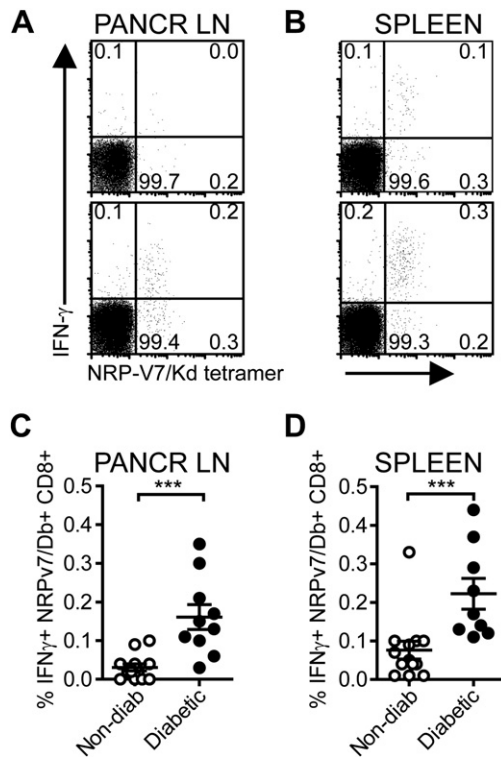
Earlier reports explain the diabetes prevention by FL treatment of young NOD mice by the associated elevated DC levels, the selective expansion of CD8 $\alpha^+$  DCs and plasmacytoid DCs (pDC) [8,23], or the induction of Treg [14]. Here, we confirm these observations in young mice for DC subsets (Fig. 2A) and Treg (Fig. 2B). We anticipated that late stage FL treatment lost the capacity to increase the levels of DC and Treg, but found that DC levels were still strongly elevated by late stage FL treatment, albeit slightly less dramatically than early treatment (Fig. 2). This is possibly because CD135 expression is slightly reduced in late versus early stage diabetes development on pDCs and to lesser extent on CD8 $\alpha^+$  DCs and mDCs (Supplementary Fig. 1). Furthermore, we found that both early and late stage FL treatment selectively increases FoxP3 $^+$  CD4 Treg levels in spleen, pancreatic LN and blood, albeit somewhat less striking in the periphery of 15 week old mice (Fig. 2B). CD8 T cells are important for the destruction of islets in NOD mice [24,25]. It is however not known whether FL affects CD8 T cell levels or function. We found here that late stage FL treatment elevated CD8 numbers somewhat more than early stage treatment in both pancreatic LN ( $2.50 \pm 0.65$  vs  $1.83 \pm 0.38 \times 10^6$  cells) and spleen ( $1.66 \pm 0.22$  vs  $1.58 \pm 0.31 \times 10^6$  cells) (Fig. 2B). Interestingly, while in both young and old mice bulk Treg outnumber NRP-V7-specific autoaggressive CD8 T cells, we found that the ratio of autoaggressive CD8 T cells to Treg increased dramatically in both spleen and pancreatic LN as NOD mice age (Fig. 2C). This altered balance of islet-aggression versus counter-regulation in favor of the autoimmunity warranted further investigation (see below). Taken together, our data show that the reported expansion of DCs and induction of Treg is not significantly impaired

**Fig. 2.** Elevation of DC and Treg numbers by FL treatment is not significantly different comparing intervention at early versus later stages of diabetes development. Female NOD mice were treated with 10 daily doses of 10  $\mu$ g huFL i.p. (black bar) or carrier (control, white bars) at 6 weeks ( $n = 3$ /group) or 15 weeks ( $n = 10$ /group) of age as indicated. On day 14 after start of treatment, spleen, pancreatic LN and blood were analyzed. A: DC analysis; displayed are cell numbers of CD8 $\alpha^+$ DC (CD8 $\alpha^+$ CD11c<sup>High</sup>B220<sup>-</sup>CD24<sup>+</sup>CD172<sup>-</sup>CD3<sup>-</sup>), myeloid DC (mDC, CD11c<sup>High</sup>B220<sup>-</sup>CD24<sup>-</sup>CD172<sup>-</sup>CD3<sup>-</sup>, all CD8 $\alpha^+$ ), plasmacytoid DCs (pDC, CD11c<sup>Int</sup>B220<sup>+</sup>CD8 $\alpha^+$ CD3<sup>-</sup>) in spleen of control- (open bars) or FL-treated (black bars) NOD mice of 6 or 15 weeks old. B: T cell analysis; displayed are conventional CD4 T cells (Tconv, CD4<sup>+</sup>CD3<sup>+</sup>FoxP3<sup>-</sup>CD8<sup>-</sup>), regulatory CD4<sup>+</sup> T cells (Treg, CD4<sup>+</sup>CD3<sup>+</sup>FoxP3<sup>+</sup>CD8<sup>-</sup>), CD8 T cells (CD8<sup>+</sup>CD3<sup>+</sup>CD4<sup>-</sup>) in spleen (top panel, cell numbers), pancreatic LN (middle panel, cell numbers) and blood (bottom panel, frequency) of control- (open bars) or FL-treated (black bars) NOD mice of 6 or 15 weeks old. C: Ratio of NRP-V7/Kd tetramer-positive CD8 T cells versus Treg in spleen (left panel) and pancreatic LN (right panel) at 6 wks and 15 wks of age. Statistical significance was calculated by *t*-test: (\*\*\*)  $p < 0.001$ , (\*\*)  $p < 0.01$ , (\*)  $p < 0.05$ .

after late stage FL treatment, but the increased expansion of CD8 T cells following treatment of older NOD mice warranted further investigation.

### 3.3. IGRP-specific CD8s in the blood determine outcome of FL treatment

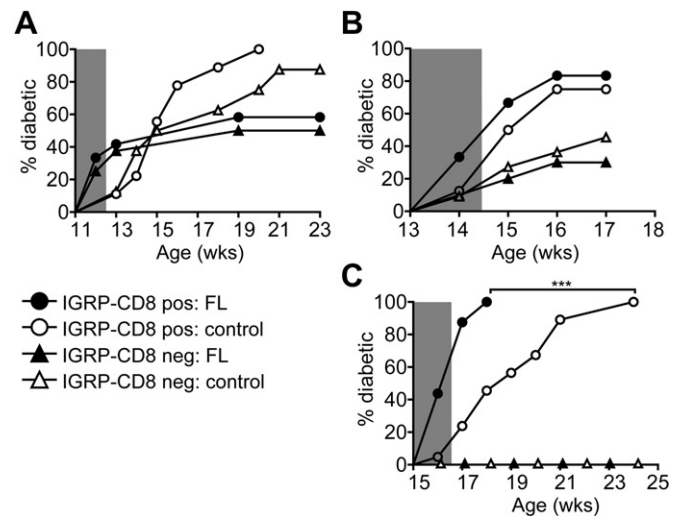
Diabetes development and onset are not fully synchronized in NOD cohorts. Thus, we reasoned that the presence or absence of ongoing islet autoimmunity and inflammation could underlie the observed dual outcome of FL treatment at later stages of diabetes. We had already observed that total CD8s were increased by late stage FL treatment. Here, we wanted to test the hypothesis that the presence of auto-antigen specific T cells would predispose for developing accelerated T1D after FL treatment and thus could function as a biomarker. The tools to track auto-antigen specific T cells in NOD diabetes are limited, but H2-K<sup>d</sup> MHC Class I tetramers complexed to NRP-V7 peptide allow the identification of islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)-specific autoreactive CD8 T cells, which can function as a good indicator for the progression of T1D in the NOD [21,22,26]. In a first approach, we determined IGRP-specific CD8 functional responses in NOD mice that had turned diabetic versus remained normoglycemic after late stage FL treatment. Interestingly, mice



**Fig. 3.** Protection or acceleration of diabetes by FL treatment in NOD mice is associated with less or more IFN- $\gamma$  producing IGRP-specific CD8 T cells, respectively. Female NOD mice (13–15 weeks old) were treated with 10 daily doses of 10  $\mu$ g FL i.p. At diabetes onset, or four weeks post treatment, IFN- $\gamma$  production by IGRP-specific autoreactive CD8 T cells in the pancreatic LN or spleen was determined by staining with NRP-V7/Kd tetramer before *in vitro* restimulation with NRP-V7 peptide for 5 h in the presence of Brefeldin A and ICCS. **A, B:** Representative dot plots of CD8 T cell gate, displayed as NRP-V7/Kd tetramer versus IFN- $\gamma$  staining from **A:** Pancreatic LN and **B:** Spleen for non-diabetic (top) or diabetic (bottom) mice. Indicated values are percentage of CD8, rounded to nearest first decimal. **C, D:** Results were grouped into non-diabetic (Non-diab,  $n = 13$ ) and diabetic ( $n = 10$ ) mice as indicated and the data displayed are the percentage of IFN- $\gamma$ + NRPV7/Kd+ CD8 T cells in the lymphocyte gate (**C:** Pancreatic LN, **D:** Spleen). Statistical analysis was done by Mann–Whitney *t*-test: (\*\*\*)  $p < 0.001$ . Representative dot plots of NRP-V7/Kd tetramer versus IFN- $\gamma$  staining, gated on CD8 T cells.

that had rapidly turned diabetic upon FL treatment contained a significantly higher number of IFN- $\gamma$  producing IGRP-specific CD8 T cells than FL mice that remained non-diabetic within the time frame of the study (Fig. 3). We observed this in both spleen and pancreatic LN. These data suggest a link between the presence of IGRP-specific CD8 T cells and the rapid onset of diabetes after FL treatment during later stages of diabetes development and imply that FL-enhanced DCs would be better able to propagate auto-reactive CD8 responses.

Our second approach was to test whether FL would accelerate disease in mice with pre-existing CD8 autoreactivity, but delay T1D onset in mice without detectable CD8 autoreactivity. It is known that even within age-matched groups, each individual NOD mouse exhibits a unique distribution and asynchronous appearance of  $\beta$ -cell-reactive CD8<sup>+</sup> T cells [27]. To address this, we segregated NOD mice at several ages based on the presence of IGRP-specific CD8 T cells. We measured the frequency of IGRP-specific CD8 T cells in peripheral blood on a weekly basis starting at week 9 until the inception of treatment. A single frequency of >0.50% of the CD8<sup>+</sup>CD19<sup>-</sup> cell population or a cumulative frequency of >0.75% over the test period characterized mice for inclusion in the 'IGRP-CD8 pos' group, while the others were assigned to the 'IGRP-CD8 neg' group (also see Methods and Supplementary Fig. 2). Crucial was thus the segregation based on the presence of IGRP-CD8 and the non-diabetic status of the mice within the screening period. Next, mice were randomized for treatment with FL or carrier control, and monitored for diabetes onset (Fig. 4). At younger ages, when FL treatment is protective, IGRP-specific CD8 T cells were hardly



**Fig. 4.** FL treatment accelerates diabetes only in NOD mice containing detectable IGRP-specific autoreactive CD8 T cells in blood. Female NOD mice were pre-screened weekly for the presence of IGRP-specific (NRP-V7/H-2K<sup>d</sup> tetramer<sup>+</sup>) autoreactive CD8<sup>+</sup> T cells in the blood starting at week 9 until the start of treatment (see Methods, Supplementary Fig. 2, and Ref. [22]); mice treated at week 11, 13, or 15 were screened weekly at week 9–11, 9–13, 9–15, respectively). At the start of treatment, the mice with a single measurement of >0.5% or with a cumulative frequency of more than 0.75% NRP-V7/Kd tetramer<sup>+</sup> cells in the CD8<sup>+</sup>CD19<sup>-</sup> lymphocyte gate over all performed screenings were grouped as 'IGRP-CD8 pos'. Within the 'IGRP-CD8 pos' (circles) and 'IGRP-CD8 neg' (triangles) groups, normoglycemic mice were randomized and treated for 10 consecutive days (shaded area) with 10  $\mu$ g i.p. huFL (black symbols) or carrier solution (open symbols) starting at **A:** 11 weeks, **B:** 13 weeks or **C:** 15 weeks of age. Blood glucose values were monitored before, during (shaded area) and after treatment. Diabetes onset was dated on the first of 2 consecutive readings >200 mg/dl. Shown is the diabetes incidence in each group. Statistical significance was determined by log-rank Mantel–Cox: (\*\*\*)  $p < 0.001$ . Number of mice: 11 weeks (IGRP-CD8 pos FL,  $n = 12$ ; IGRP-CD8 pos control,  $n = 9$ ; IGRP-CD8 neg FL,  $n = 8$ ; IGRP-CD8 neg control,  $n = 9$ ), 13 weeks (IGRP-CD8 pos FL,  $n = 6$ ; IGRP-CD8 pos control,  $n = 8$ ; IGRP-CD8 neg FL,  $n = 10$ ; IGRP-CD8 neg control,  $n = 11$ ), 15 weeks (IGRP-CD8 pos FL,  $n = 16$ ; IGRP-CD8 pos control,  $n = 21$ ; IGRP-CD8 neg FL,  $n = 5$ ; IGRP-CD8 neg control,  $n = 5$ ).

detectable in blood (data not shown). At 11 weeks of age, both FL and control treatment resulted in a slight trend of accelerated diabetes onset in the 'IGRP-CD8 pos' versus the 'IGRP-CD8 neg' group (Fig. 4A). However, at this age we did not find a significant correlation between the presence of IGRP-specific CD8s and the outcome of FL treatment. Indeed, FL treatment accelerated T1D in a similar fraction of the 'IGRP-CD8 pos' group and the 'IGRP-CD8 neg' group (Fig. 4A). At 13 weeks, IGRP-specific CD8s in the blood become more accurate at predicting diabetes onset (Fig. 4B and Ref. [22]). At that age, carrier control-treated 'IGRP-CD8 pos' mice developed diabetes faster than 'IGRP-CD8 neg' controls (Fig. 4B). More important, FL treatment accelerated diabetes development only in the 'IGRP-CD8 pos' NOD mice, while a slight trend towards delayed onset was observed in 'IGRP-CD8 neg' mice (ns) (Fig. 4B). At 15 weeks, FL treatment dramatically accelerated diabetes onset in pre-diabetic 'IGRP-CD8 pos' NOD mice as compared to control-treated 'IGRP-CD8 neg' mice (Fig. 4C). As a matter of fact, the consequences of FL treatment under conditions of pre-existing autoimmunity and islet inflammation were rather severe: not only did the majority of mice develop rapid diabetes, about 25% of the mice had to be euthanized during the treatment regimen due to poor health status under hyperglycemia (data not shown). At 15 weeks it was not possible to determine whether FL confers extra protection to 'IGRP-CD8-neg' NOD, because none of the control-treated 'IGRP-CD8 neg' mice developed diabetes by 25 weeks of age, while age-matched control-treated 'IGRP-CD8-pos' NODs were all diabetic by that time (Fig. 4C). In summary, based on our data reported here, there is a strong correlation between pre-existing CD8 autoreactivity and accelerated autoimmune diabetes onset after FL treatment.

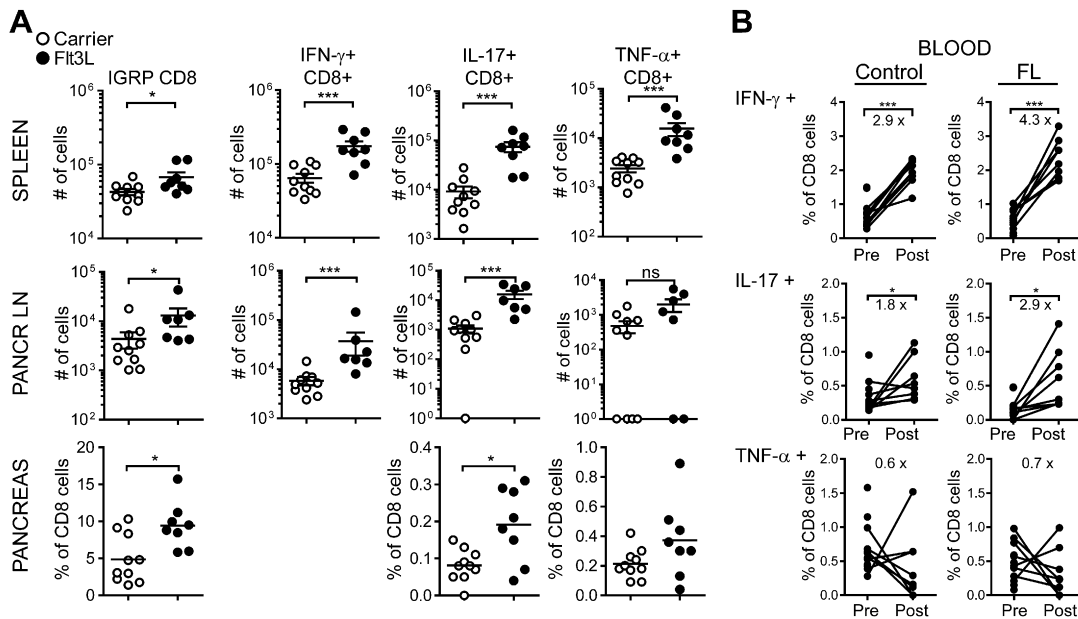
3.4. FL accelerates disease by enhancing pre-existing CD8 autoreactivity

Next, we determined whether FL treatment accelerated diabetes in NOD mice with pre-existing CD8 autoreactivity by potentiating

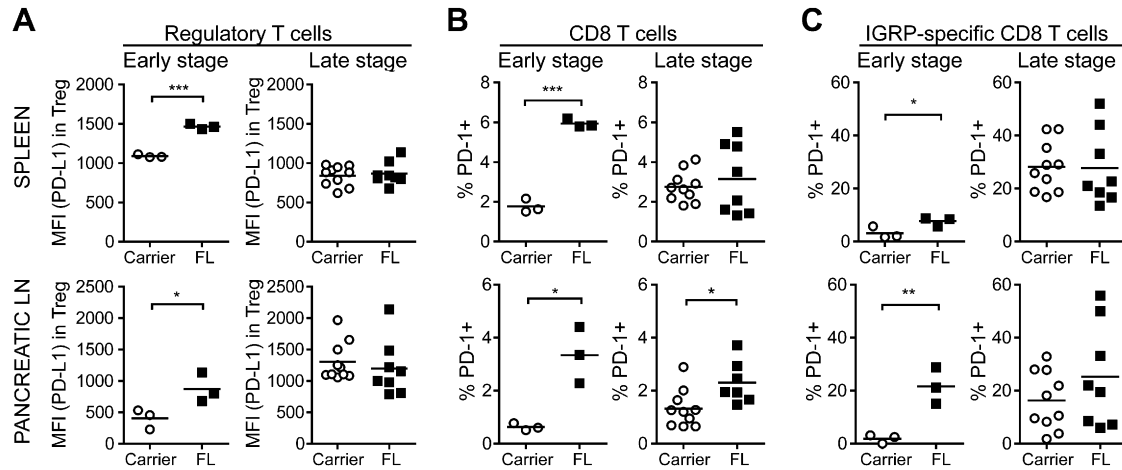
the numbers and/or function of the IGRP-specific CD8s. To test this, we screened weekly (wk 9–15) for IGRP-specific CD8 T cells and treated 15 week old non-diabetic 'IGRP-CD8 pos' NODs with FL or carrier control. This allowed us to focus only on mice that would become diabetic, but faster upon FL treatment. Next, we determined the presence and functional response of the IGRP-specific CD8 T cells in each mouse (Fig. 5). We found that FL significantly increased the number of IGRP-specific CD8s in spleen, pancreatic LN and pancreas (Fig. 5A, left panels). Moreover, the relative fraction of CD8 T cells (data not shown) or the total numbers of CD8 T cells producing TNF- $\alpha$ , IL-17, or IFN- $\gamma$  upon in vitro IGRP-peptide restimulation was increased in spleen and pancreatic LN (Fig. 5A, right panels). Likewise in the pancreas, we found significantly higher production of IL17 and a trend towards increased TNF- $\alpha$  production (Fig. 5A). In blood, a longitudinal follow-up of individual mice revealed an increase in IGRP-specific functional responses after both carrier control and FL treatment (Fig. 5B). This not only shows that normal progression of the diabetogenic process in NOD mice is associated with increased numbers and functionality in IGRP-specific CD8 T cells, in agreement with earlier data [22]. Most importantly, the fold increase in the functional response was greater in FL-treated animals, as assessed by IL-17 and IFN- $\gamma$  production (Fig. 5B, see inset values for fold increase). This indicates that FL treatment increases the function of IGRP-specific CD8s more than the diabetogenic process itself, which is also in line with the data from spleen, pancreatic LN and pancreas. Taken together, the accelerated diabetes onset in FL-treated NODs with pre-existing autoreactivity is associated with increased numbers and function of autoreactive CD8 lymphocytes.

3.5. Early but not late-stage in vivo FL treatment upregulates PD-1 on CD8 T cells and PD-L1 on Treg

The interaction of the inhibitory accessory molecule programmed death-1 (PD-1) [28] and its ligands, PD-L1 and PD-L2,



**Fig. 5.** FL increases the numbers and functional response of IGRP-specific CD8 T cells. Non-diabetic 15 week old NOD mice, screened positive for NRP-V7K<sup>d</sup> tetramer<sup>+</sup> cells, were treated with 10 daily i.p. doses of 10  $\mu$ g FL (black symbols,  $n = 8$ ) or carrier control (open symbols,  $n = 10$ ). **A:** Four days after the treatment course, cells from spleen (top row), pancreatic LN (middle row) and pancreas (bottom row) were stained with NRP-V7/K<sup>d</sup> tetramer or in vitro restimulated with NRP-V7 peptide for 5 h in the presence of BFA to measure IFN- $\gamma$ , TNF- $\alpha$  and IL-17 production by ICCS. For spleen and pancreatic LN, cell numbers of indicated population are displayed; for pancreas and blood, percentage of CD8 T cells is depicted. **B:** Before and four days after the treatment course, the frequency of IFN- $\gamma$ , TNF- $\alpha$  and IL-17 producing CD8s in blood was assayed instantaneous at time of collection by ICCS after in vitro restimulation with NRP-V7 peptide and T-cell depleted NOD splenocytes for 5 h in the presence of BFA. Depicted is the percentage of CD8 T cells positive for the indicated cytokine, the connecting lines pair values before and after treatment per mouse. The inset value reflects the fold increase of the averaged frequencies before and after treatment course. Statistical analysis for A and B was performed by unpaired and paired *t*-test, respectively: (\*\*\*)  $p < 0.001$ , (\*\*)  $p < 0.01$ , (\*)  $p < 0.05$ .



**Fig. 6.** Early but not late stage *in vivo* FL treatment upregulates the inhibitory receptor PD-1 on CD8 T cells and its ligand PD-L1 on Treg. Female pre-diabetic NOD mice were treated at 6 weeks (early stage, IGRP-specific CD8s below threshold) or at 15 weeks (late stage, IGRP-specific CD8s above threshold) with 10 daily i.p. doses of 10  $\mu$ g huFL (black symbols) or carrier control (open symbols). Four days after the treatment course, cells from spleen (top row) or pancreatic LN (bottom row) were harvested and stained. **A:** Expression of PD-L1 by regulatory T cells. Median Fluorescence Intensity (MFI) of PD-L1 expression minus isotype MFI (background) on FoxP3<sup>+</sup> CD4<sup>+</sup> T cells is displayed. **B:** Frequency of PD-1 positive cells within CD8 T cells. **C:** Frequency of PD-1 positive cells within IGRP-specific CD8 T cells. Statistical analysis was performed by Mann–Whitney non-parametric *t*-test: (\*\*\*)  $p < 0.001$ , (\*\*)  $p < 0.01$ , (\*)  $p < 0.05$ .

controls autoimmune T cell responses, notably in T1D [29]. Moreover, it was recently shown that the PD-L1/PD-1 pathway is involved in the suppression of CTL responses [30] and alloimmune responses [31]. Therefore, we assessed whether differences in expression of PD-1 and/or PD-L1 would correlate with the differential outcome comparing early versus late stage FL treatment. First, we found increased levels of surface PD-L1 on FoxP3<sup>+</sup> CD4<sup>+</sup> Treg in blood (data not shown), spleen and pancreatic LN when FL treatment was started early and acts protective (Fig. 6A). In contrast, PD-L1 levels remained unaltered on Treg after late stage treatment of 'IGRP-CD8 pos' mice (Fig. 6A), a scenario in which FL treatment accelerates diabetes. Second, the proportion of PD-1 receptor expressing CD8 T cells in blood (data not shown), spleen and pancreatic LN is increased when treatment is started early (Fig. 6B). In contrast, late stage treatment of 'IGRP-CD8 pos' NOD mice does not result in such a strong increase in PD-1 expression, and only some of the pancreatic LN CD8 T cells gain expression of PD-1 (Fig. 6B). Third, a higher fraction of IGRP-specific diabetogenic CD8 T cells expressed PD-1 when FL treatment was started early, i.e. before the IGRP-CD8 T cells reached the detection threshold in blood (Fig. 6C). In contrast, late stage FL treatment of 'IGRP-CD8 pos' mice does not further increase the fraction of PD-1 expressing IGRP-specific CD8 T cells. Taken together, our observations suggest that early but not late stage FL treatment of NOD mice renders diabetogenic CD8 T cells susceptible to PD-1-mediated inhibition via PD-L1 on Treg.

#### 4. Discussion

Our data show that Flt3-ligand treatment during advanced diabetes development stages can augment pre-existing CD8 autoreactivity, thereby accelerating diabetes in the NOD, rather than protecting from it, as had been previously reported for very early stages of T1D [8,14]. As a result, one would expect that FL treatment will not be beneficial at disease stages that correspond with the usual clinical diagnosis of type 1 diabetes in humans. FL can also not cure recent-onset diabetes (Fig. 1D), but we confirmed that FL treatment can prevent diabetes when administered early during the diabetogenic process (Fig. 1A) [8,14,20]. More importantly, we found that late stage treatment exacerbated disease in one part of the cohort, namely those mice that already harbored

pre-existing autoreactive CD8 lymphocytes, while delaying onset in those mice without detectable autoreactive CD8 T cells. In contrast to our observations, earlier data by O'Keefe et al. [8] had shown a slight protection by late stage murine FL treatment. In another study, Cheatem et al. [20] might have overlooked an acceleration of diabetes by murine FL administration at 11–13 or 15–17 weeks, because they removed the mice that were diabetic at the start of treatment only from the treatment groups, but not from the control groups, thereby impeding a clear comparison. Our findings were essentially the same when dating diabetes incidence as the first of 2 consecutive readings >250 mg/dl (data not shown) instead of >200 mg/dl (Fig. 1). Therefore, different cut-off values do not explain the differences between the study by Cheatem et al. [20] and ours. Also, different "cleanliness" status of the colonies [32] might be responsible for the diverging incidence and rate of diabetes development. However, we found a dual effect of FL within the same cohort, indicating that this differential effect is a function of the stage of diabetes progression in individual mice, and not due to environmental factors affecting the colony.

We also observed that the two previously described effects of early FL treatment in NOD mice, i.e. elevation of DC and Treg numbers, were not significantly impaired following late stage treatment. We therefore concluded that another mechanism must be the cause for the acceleration as well as the delay observed after late stage treatment. It is known that even within age-matched groups, each individual NOD mouse exhibits a unique distribution of  $\beta$ -cell-reactive CD8<sup>+</sup> T cells, and that the inflammatory process in each individual follows its own distinctive course [27]. Here, we found that those mice that rapidly became diabetic upon late stage FL treatment contained a higher number of IFN- $\gamma$  producing autoreactive, diabetogenic IGRP-specific CD8 T cells, as compared to those that remained non-diabetic for a longer time (Fig. 3). Moreover, NOD mice that contained pre-existing IGRP-specific CD8s in the blood before treatment at 13 and 15 weeks of age developed diabetes faster upon subsequent FL administration than mice that did not contain detectable levels of these autoreactive CD8s (Fig. 4). Such a dichotomy in outcome was not observed at 11 weeks of age, likely because of the technical limits of the screening for IGRP-specific CD8 T cells. Indeed, given the fluctuation of the IGRP-specific CD8 frequency in blood, more and prolonged screening (only 3 for 11 weeks, but 7 for 15 weeks) increases the chance that

IGRP-specific CD8s are present above the detection limit. It is likely that the inclusion of other CD8 specificities would yield better inclusion/exclusion criteria. In NOD mice, the insulin-reactive G9C8-like T cells (INS-L9/H-2K<sup>d</sup>) [33], 8.3-like T cells and AI4-like T cells (MimA2/H-2D<sup>b</sup>) [34], often account for a considerable proportion (up to 60%) of the islet infiltrating CD8 T cells [21,22,27,35,36]. The fact that IGRP-specific CD8 T cells in the blood are under the threshold at early stages (data not shown and Ref. [22]) further supports the concept that the presence of autoreactive CD8s determines the diabetes outcome of FL treatment. Nevertheless, when IGRP-specific CD8 T cells are detectable in blood, FL treatment doubles their number and increases their functional response 10-fold as compared to control treatment. Control treatment itself reveals that numbers and IFN- $\gamma$  production of autoreactive CD8 increase during the NOD diabetogenic process (Fig. 5). An alternative explanation could be that the appearance of autoreactive CD8 T cells marks a point of intense inflammation that could activate the DCs generated by FL treatment, shifting them from a tolerogenic to an immunogenic state. Blockade of the interaction of PD-1 and its ligands rapidly precipitates diabetes in NOD [29]. Although to our knowledge no comprehensive analysis of PD-1 and PD-L1 molecules on CD8, effector and regulatory CD4 T cells during diabetes development in the NOD mouse has been reported, NOD CD8 T cells upregulate programmed death-1 (PD-1) upon activation [37] and PD-L1 levels on T cells wane as NOD mice age [29]. Moreover, CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells employ the PD-L1/PD-1 pathway during suppression of CTL responses [30] and alloimmune responses [31]. Here, we found that early stage FL treatment upregulates the inhibitory receptor PD-1 on (IGRP-specific) CD8 T cells and its ligand PD-L1 on regulatory T cells (Fig. 6). Upregulation of this inhibitory pathway no longer happens when the islet-specific CD8 T cells become detectable in the blood. It was recently shown that regulatory T cells are required for FL-mediated suppression of T1D in young NOD mice [38]. In light of the data on the functional waning of regulatory T cells with age in NOD mice [39], our data suggests that, although both early and late stage FL treatment expanded Tregs (Fig. 2), FL treatment failed to further increase PD-L1 expression on Tregs (Fig. 6A), which might have repercussions on their suppressive capacity.

Perhaps, an adverse outcome of late stage FL treatment could be curbed by combination therapies, aimed at increasing Treg functionality and/or silencing autoreactive CD8 responses.

## 5. Conclusion

Two main issues emerge from our findings. A mere increase in DC subsets does not delay the diabetogenic process in the NOD. Therefore, therapies targeting DCs should probably better aim at generating and/or stimulating tolerogenic DCs rather than just augmenting their numbers. One such example is the use of immunoregulatory dendritic cells (iDC), autologous monocyte-derived DCs in which the CD40, CD80 and CD86 co-stimulatory molecules are downregulated by antisense molecules before reintroduction. An approved clinical safety study (NCT00445913) will evaluate the success achieved in NOD mice [40,41]. Second, from a clinical standpoint it will prove useful to pre-screen patients for a specific type of treatment based on the presence or absence of certain 'biomarkers', for example autoreactive T cells. Current identification uses: genetic screening, by relationship to diabetic individual or identification of high-risk HLA genotypes, serologic screening for serum autoantibodies associated with islet  $\beta$ -cells (GAD65, ICA512, and insulin) [42], and metabolic testing of low first phase insulin production and/or impaired fasting glucose or impaired glucose tolerance [43,44]. In addition, our study supports the use of peripheral blood as source of biomarkers, such as pre-

existing T cell autoreactivity in screening efforts to reveal additional events that reflect disease activity. In humans, epitopes from GAD65 [45], human islet amyloid polypeptide (IAPP) precursor protein [46], preproinsulin signal peptide [47], and cation efflux transporter ZnT8 (Slc30A8) [48] can be considered for pre-screening to more precisely determine disease activity [36]. Such a strategy might also be of importance for choosing the optimal antigen for induction of Tregs in antigen specific intervention trials. In conclusion, adding T cell autoreactivity to a panel of biomarkers to compile a more complete picture of disease activity should help in making treatment of autoimmune disease better and safer.

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## Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jaut.2009.11.010](https://doi.org/10.1016/j.jaut.2009.11.010).

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