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Functional cytotoxic T lymphocytes against IGRP$_{206-214}$ predict diabetes in the non-obese diabetic mouse

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Running title: IGRP-specific CTLs predict NOD diabetes

Abstract

$CD8^+$ T cells are prominent in autoimmune diabetes of both humans and non-obese diabetic (NOD) mice. For example, $CD8^+$ T cells against islet-specific glucose 6-phosphatase catalytic subunit-related protein (IGRP) can be detected readily in older NOD mice. It has been suggested that the enumeration of islet-specific $CD8^+$ T cells in the peripheral blood may be a predictive biomarker for autoimmune type 1 diabetes (T1D). Here, we determined the natural history of the functional endogenous $IGRP_{206-214}$-specific cytotoxic T lymphocytes (CTLs) in NOD mice with regards to age (3 to 15 week old pre-diabetic mice and diabetic mice) and sex. We demonstrated that *in vivo* $IGRP_{206-214}$-specific CTLs significantly increased after 12 weeks of age and *in vivo* cytotoxicity in female NOD mice was significantly higher than in male NOD mice. To determine the *in vivo* $IGRP_{206-214}$-specific CTL frequency without killing the mice, we performed splenectomies on a cohort of mice after injecting $IGRP_{206-214}$-coated targets and then followed their diabetes progression. We found that CTL frequency correlated with future of disease onset. Thus, our data support that $IGRP_{206-214}$-specific CTLs may be a potent biomarker for T1D.

Introduction

Type 1 diabetes (T1D) results from T cell-mediated autoimmune destruction of insulin-producing beta cells of the pancreas.[1] $CD8^+$ T cells are dominant in islet infiltrates in human T1D.[2-4] Although $CD4^+$ T cells have an influential role in NOD diabetes,[5-7] the evidence for a pivotal role of $CD8^+$ T cells is compelling. Perforin deficient and MHC I deficient NOD mice have reduced disease and both $CD4^+$ and $CD8^+$ T cells are required for efficient adoptive transfer of disease.[8-11] Moreover, many candidate T1D autoantigens educe CTL responses, including insulin, glutamic acid decarboxylase, islet-specific glucose 6-phosphatase catalytic subunit-related protein (IGRP) and insulinoma-associated antigen 2.[12-15] These antigen-specific CTLs can be prevalent within the islets of pre-diabetic NOD mice and $IGRP_{206-214}$-specific $CD8^+$ T cells are highly diabetogenic.[16 17] Moreover, IGRP-specific $CD8^+$ T cells have been histologically identified in islets of deceased people with T1D.[4]

In humans, development of antibody responses to multiple antigens is predictive of disease.[18] Despite the pathogenic role of $CD8^+$ T cells, few studies utilized them to predict diabetes by the detection of their frequency in peripheral blood of NOD mice or human patients.[19-21] One study in particular indicated that enumeration of circulating tetramer-specific $CD8^+$ T cells has predictive value for disease[19]. Such predictions may avail better tailoring of therapeutic intervention.

We wondered whether a functional CTL read-out (in contrast to tetramer enumeration) might indicate ongoing disease. Therefore, we first quantified the natural history of the functional endogenous $IGRP_{206-214}$-specific CTLs in NOD mice with regard to age and sex by using an *in vivo* CTL killing assay.[22] We then determined whether *in vivo* $IGRP_{206-214}$-specific CTLs were correlated with future disease onset.

RESULTS AND DISCUSSION

Functional cytotoxic IGRP$_{206-214}$-specific CTLs can be detected *in vivo* before the onset of disease and increase with age.

IGRP$_{206-214}$-specific CD8+ T cells can be identified by tetramer staining in NOD mice. IGRP$_{206-214}$-specific CTLs have also been shown ex vivo after in vitro expansion. However, whether endogenous IGRP$_{206-214}$ specific functional killers are generated *in vivo* has still not been established. Therefore, we investigated the natural history of the functional endogenous IGRP$_{206-214}$-specific CTLs in NOD mice (spontaneous diabetic mouse model) by using the *in vivo* CTL assay. We analyzed female NOD mice from 3 weeks to 15 weeks of age, newly diabetic mice (two weeks after onset of diabetes) and long-term diabetic mice (more than two weeks after onset of diabetes). Target cells specific for IGRP$_{206-214}$ CTLs were generated by using donor splenocytes from NOR/Lt mice (MHC-matched diabetes-resistant strain)[23] pulsed with IGRP$_{206-214}$ peptides and control cells were pulsed without peptides. They were distinguished by different CFSE fluorescent intensity: target cells being CFSE^{hi} and control cells being CFSE^{lo}. Specific lysis of target cells was reflected by a decrease in the ratio between the CFSE^{hi} and ^{lo} cells in NOD mice compared with the ratio in NOD-PI mice (Figure 1a). NOD-PI mice served as good negative controls as the transgenic expression of preproinsulin II under the MHC II promoter abrogated diabetes.[24] To detect *in vivo* killing of target cells by the endogenous CTLs, pancreatic lymph nodes (PLNs) and spleens were analyzed at 18-20 h after injecting target cells. We observed that cytotoxicity of IGRP$_{206-214}$-specific *in vivo* CTLs in PLNs appeared from 9-11 weeks of age and significantly increased between 12-15 weeks ($p<0.0001$; Figure 1b) in pre-diabetic NOD mice. The highest specific lysis by IGRP$_{206-214}$ CTLs was found in mice with diabetes of recent onset. This may have been due to higher levels of autoantigens being released from the many islets being attacked, whereas later when many of the islets have already been eliminated, antigen became less

available. Killing in spleen was similar to that in PLNs (data not shown); the expected 3-day delay between LN priming and subsequent seeding to the spleen would not necessarily be conspicuous in this study, as priming would not be expected to be synchronous within a cohort of mice.

Diabetes is more prevalent in female than male NOD mice. Therefore, we investigated the sex difference of $IGRP_{206-214}$-specific CTL activity and examined *in vivo* CTLs with age-matched NOD cohorts (Female, n=43; Male, n=45). We found that pre-diabetic NOD female mice (15 weeks) showed more killer activity than male mice in PLNs (Figure 1c, p=0.0024) as well as in spleen (data not shown).

We further clarified that the levels of $IGRP_{206-214}$-specific killing *in vivo* correlated with numbers of tetramer positive $CD8^+$ T cells. Spleen cells from 15 week-old NOD mice (n=16) that were injected with target cells were individually stained with $IGRP_{206-214}$-specific tetramer. This allowed evaluating both cytotoxicity and numbers of $IGRP_{206-214}$-specific CTLs from the same mouse. As expected, numbers of tetramer positive cells in 15 week-old NOD mice were 35-fold higher than in non-diabetic NOD-PI mice (Figure 1d, upper). There was also a significant correlation between numbers of tetramer positive cells and *in vivo* cytotoxicity (Figure 1d, lower).

High *in vivo* CTL killing correlates with subsequent diabetes onset in individual mice

Given that $IGRP_{206-214}$-specific *in vivo* CTLs were detectable in spleen from pre-diabetic NOD mice, we examined whether *in vivo* CTL detected from spleen in mice predicted subsequent disease onset individually. To do this we had to perform splenectomies to enumerate killing of dye-labeled targets without killing the mice so that we could later determine their diabetes incidence. Others had already shown that splenectomy did not alter the course of NOD diabetes.[25] We splenectomized mice (15 weeks of age (about 100 days)) at

one day after injection of target cells for *in vivo* CTL and subsequently monitored the incidence of diabetes (Figure 2). Killing by $IGRP_{206-214}$-specific *in vivo* CTLs was analyzed from individual splenectomised NOD female mice (n=55) together with 10 week-old NOD female mice (n=9) and non-diabetic NOD-PI mice (n=11) (Figure 2a). As we have shown in Figure 1, 15 week-old NOD female mice showed various levels of $IGRP_{206-214}$-specific lysis (0 ~ 60%). We divided the splenectomized mice in two groups – the mice that were positive for $IGRP_{206-214}$-specific *in vivo* CTL (specific lysis >10%, n=30) and the negative group (specific lysis <10%, n=25) and then monitored their diabetes onset (Figure 2b). The incidence of diabetes was low in our NOD cohort. Over the course of 300 days, 18 of 55 splenectomized female mice developed diabetes at an incidence of 33%, whereas 20 of 48 age- and sex-matched intact mice showed an incidence of 42% (Figure 2c). The incidence of diabetes from the two groups was not significantly different, confirming that splenectomy had no effect on diabetes prevalence as previously reported.[25]

Of the 18 splenectomized mice that subsequently developed diabetes, 17 mice from the CTL positive group developed diabetes and only one mouse from the negative group did, resulting in a predictive value of 94.4 %. We also found that the level of *in vivo* CTL killing was significantly correlated with diabetes onset (Figure 2d; p=0.0064). 10 mice from the CTL positive group that showed 25-60 % of specific lysis developed diabetes between the ages of 100 and 200 days, resulting in a positive predictive value of 58.8 %. In contrast, 6 mice that had the lower $IGRP_{206-214}$-specific lysis (10-25%) developed diabetes later than 200 days and showed a lower predictive value (35.3 %), respectively. Only one mouse from the unpredicted group (n=25) developed diabetes, indicating a negative prediction value of 96%.

Our data clearly indicate that $IGRP_{206-214}$-specific *in vivo* CTLs in NOD mice predict diabetes.

METHODS

Mice

NOD, NOR/Lt and NOD-PI[24] mice were bred under specific pathogen-free conditions in the animal facility of WEHI. Experiments were performed according to the guidelines of the Institute's Animal Ethics Committee. Urine glucose was monitored weekly. Mice with positive urine glucose were confirmed to be diabetic by blood glucose. Blood glucose (BG) was monitored using Advantage II Glucose Strips (Roche, Basel, Switzerland) and diabetes was defined as two consecutive reading ≥15 mM.

$IGRP_{206-214}$ specific *in vivo* cytotoxicity assay

Splenocytes were prepared from NOR/Lt mice and resuspended at 10^7 cells/ml in PBS containing 1% FCS. Cells were divided into two groups. Target splenocytes were pulsed with 2.5 μg/ml $IGRP_{206-214}$ peptide (VYLKTNVFL) for 1 h at 37°C. After washing, peptide pulsed cells were labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) ($CFSE^{hi}$) and control unpulsed cells were labeled with 0.5 μM CFSE ($CFSE^{lo}$) for 10 min at 37°C. Labeling was stopped by quenching with cold PBS containing 2.5% FCS. After washing with PBS, cells were mixed in equal proportions and 1 x 10^7 total cells in 200 μl injected intravenously into NOD and NOD-PI mice. Pancreatic lymph nodes and spleens were collected 18-20 h later and single cell suspensions were prepared. Analysis was performed on a BD LSRFortessa and data were analyzed using FlowJo software (Tree Star, Ashland, OR). Percent specific lysis was calculated as: $(1-r_{NOD-PI} / r_{NOD}) \times 100$, where $r = \%\ CFSE^{lo} / \%\ CFSE^{hi}$.

Tetramer and magnetic bead based enrichment

A tetramer and magnetic bead based enrichment method was previously described to detect low frequency naïve viral specific T cells in both humans and mice.[26] Single cell suspensions of splenocytes from individual mice were stained with $IGRP_{206-214}$ (VYLKTNVFL) H-2Kd tetramer (ImmunoID Flow Cytometry Facility, Melbourne, Australia) for 60 min at 4 °C, washed and stained with anti-PE magnetic beads (Miltenyi Biotec, Cologne, Germany) as per manufacturer's instructions. Magnetic separation was performed using an AutoMACSpro system (Miltenyi Biotec). The separated fractions were stained with anti-CD3 (145-2C11) and anti-CD8a (5H10) antibodies and analyzed by flow cytometry.

Surgical excision of splenectomy

Mice were anesthetized by Methoxyflurane Inhalation Analgesic (Medical Developments International PTY.LTD, Springvale, Australia) and injected with Carprofen (0.2 ml / 20g of mice). Mice were laid on its right side and a dorsoventral incision in the skin and muscles was made over the spleen. The spleen was exposed and blood vessels were sectioned with an ophthalmic cautery. After retraction of spleen, incisions were suturing of the abdominal wall followed by closure of the skin with a wound clip.

Statistical analysis

Mean and SEM values, One-way ANOVA with Tukey's multiple comparison test, Pearson correlation coefficients (two tailed) and the logrank test & Ghan-Breslow-Wilcoxon test for the incidence of diabetes performed with Graphpad Prism Software. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, *n.s.*= not significant.

CONFLICT OF INTEREST

The authors have no financial conflicts of interest.

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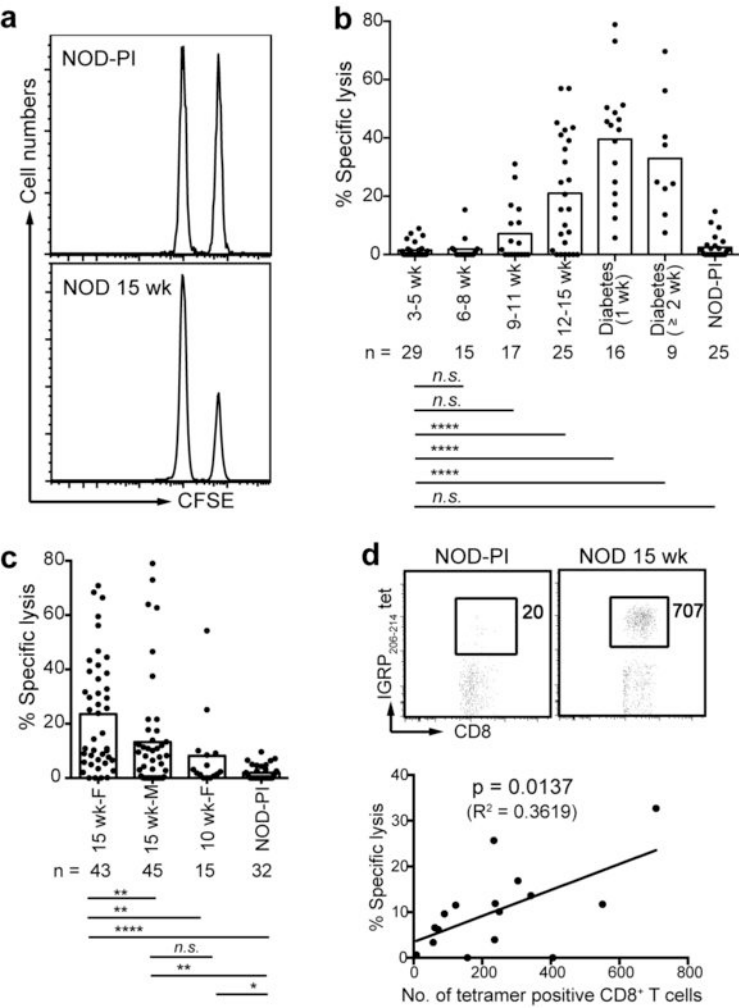
FIGURE LEGENDS

Figure 1 Natural history of $IGRP_{206-214}$-specific *in vivo* CTL in NOD mice. Target cells were prepared by pulsing splenocytes of NOR/Lt mice with or without $IGRP_{206-214}$ peptide and labeling with CFSE. Peptide pulsed cells were labeled with 5 μM CFSE ($CFSE^{hi}$) and control unpulsed cells were labeled with 0.5 μM CFSE ($CFSE^{lo}$). Cells were mixed (1:1) and injected into recipient mice. (a-c) Pancreatic LNs from individual mice were recovered 18-20 h later and analyzed by flow cytometry. Specific lysis is reflected by a decrease in the ratio between the target and control populations in NOD mice compared with the ratio in NOD-PI mice. (a) Histograms represent the population of $CFSE^{hi}$ and $CFSE^{lo}$ cells in NOD-PI and 15 week-old NOD female mice. (b) Female NOD mice from 3 weeks to 15 weeks of age, newly diabetic mice (1 week after BG ≥15 mM) and long-term diabetic mice (more than 2 weeks after BG ≥15 mM) were analyzed for the specific lysis. Data shown are combined with eight independent experiments. (c) Age of 15 weeks NOD female and male mice, 10 week-old NOD female mice and NOD-PI mice were analyzed for the specific lysis. Data shown are combined with five independent experiments. (b,c) The column indicates the mean with each dot representing an individual mouse. One-way ANOVA with Tukey's multiple comparison test was used for statistical analysis. *P<0.05, **P<0.01, ****P<0.0001, n.s.=not significant (d) Spleens from age of 15 weeks NOD mice (n=16) and NOD-PI (n=16) that were injected with target cells for 18-20 h were harvested and analyzed for specific lysis and tetramer staining. $IGRP_{206-214}$–specific $CD8^+$ T cells were enriched using the tetramer and magnetic bead method, and enumerated using flow cytometry. Dot plots (upper) display the numbers of tetramer positive $CD8^+$ T cells in spleen and the graph (lower) indicates the correlation between specific lysis and numbers of tetramer positive cells from individual mice. P value was analyzed by Pearson correlation coefficients (two-tailed).

Figure 2. Prediction of disease onset by functional $IGRP_{206-214}$-specific CTLs in NOD mice. Target cells were prepared as described in Figure 1. Specific lysis of $IGRP_{206-214}$-specific *in vivo* CTLs was analyzed in 15 week-old NOD female mice (n=55), 10 week-old NOD female mice (n=9) and NOD-PI (n=11). 15 week-old NOD mice were undergone splenectomy. Spleens from individual mice were utilized for analyzing cytotoxicity of *in vivo* CTLs and splenctomized mice were monitored for the incidence of diabetes. Data shown are combined with three independent experiments. (a) Dots in graph indicate % specific lysis of individual mice from different groups. (b) Two groups from splenectomised mice were divided by specific lysis (10 %) indicated with the solid line in (a). The $IGRP_{206-214}$-specific *in vivo* CTL positive group (specific lysis>10%, n=30, solid line) and negative group (specific lysis<10%, n=25, dot line) were monitored for the incidence of diabetes. (c) The incidence of diabetes was compared between the splenectomised mice group (n=55) and the age matched NOD female cohort mice group (without splenectomy, n=48). P value for (b) and (c) is analyzed by both the logrank test and Ghan-Breslow-Wilcoxon test. Arrows indicate the date of splenectomy. (d) The graph indicates the correlation between specific lysis and diabetes onset from the splenectomised mice that went on to develop diabetes (n=18). P value was analyzed by Pearson correlation coefficients (two-tailed).


a
NOD-PI
NOD 15 wk
Cell numbers
CFSE
b
% Specific lysis
3-5 wk
6-8 wk
9-11 wk
12-15 wk
Diabetes (1 wk)
Diabetes (≥ 2 wk)
NOD-PI
n = 29 15 17 25 16 9 25
n.s.
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15 wk-F
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IGRP206-214 tet
CD8
20
707
p = 0.0137
(R2 = 0.3619)
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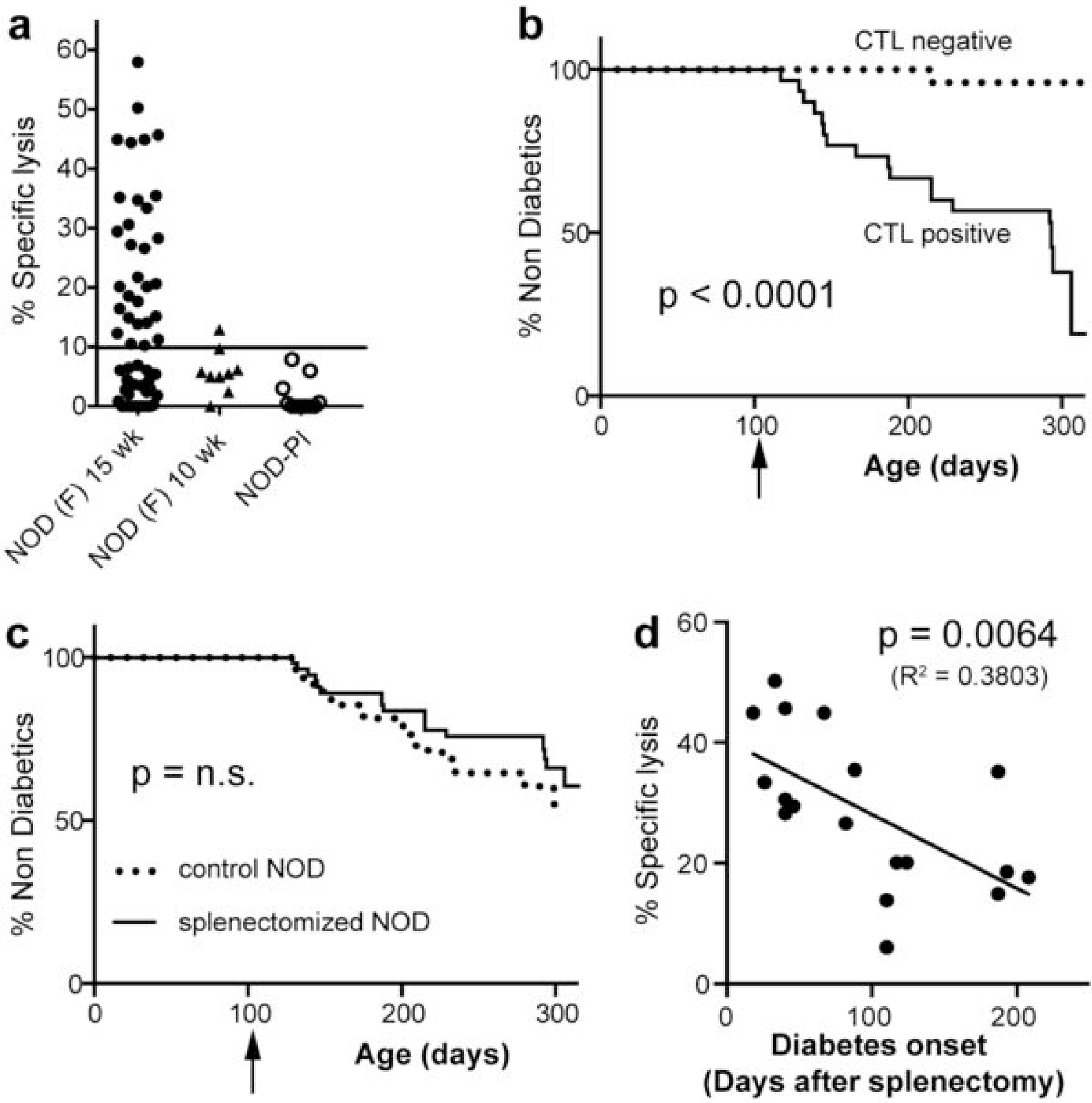
No. of tetramer positive CD8+ T cells


a
% Specific lysis
60
50
40
30
20
10
0
NOD (F) 15 wk
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NOD-PI
b
CTL negative
CTL positive
% Non Diabetics
100
50
0
p < 0.0001
0
100
200
300
Age (days)
c
% Non Diabetics
100
50
0
p = n.s.
control NOD
splenectomized NOD
0
100
200
300
Age (days)
d
p = 0.0064
(R² = 0.3803)
% Specific lysis
60
40
20
0
0
100
200
Diabetes onset
(Days after splenectomy)