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Mucosal administration of CD3-specific monoclonal antibody inhibits diabetes in NOD mice and in a preclinical mouse model transgenic for the CD3 epsilon chain

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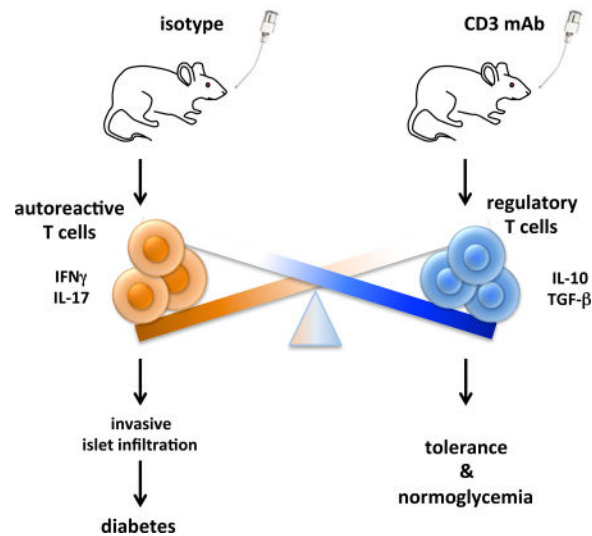
Abstract

CD3-specific monoclonal antibody (mAb) treats autoimmune disease in animal models and has shown promise in clinical trials of type 1 diabetes. Whereas intravenous administration of CD3-specific mAb acts primarily by transient depletion of activated effector T cells, oral CD3-specific mAb acts primarily by the induction Tregs. We investigated whether oral CD3-specific mAb inhibits disease in non obese diabetic (NOD) mice that spontaneously develop autoimmune diabetes, closely resembling human type 1 diabetes. We found that oral CD3-specific mAb treatment delayed onset and reduced incidence of diabetes in NOD mice, inducing changes in both effector and regulatory T cell compartments. The therapeutic effect was associated with decreased T cell proliferation, decreased IFN γ and IL-17 production, and increased TGF- β and IL-10 production *in vitro*. *In vivo* transfer experiments demonstrated that oral CD3-specific mAb decreased diabetogenicity of effector T cells and increased the function of regulatory T cells. Oral OKT3, a monoclonal antibody specific for human CD3 had equivalent effects in transgenic NOD mice expressing the human CD3 epsilon chain which serves as a preclinical model for testing human CD3-specific mAb. These results suggest that oral CD3-specific mAb has the potential for treating autoimmune diabetes in humans.

Graphical abstract

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Keywords

Autoimmune diabetes; immunotherapy; mucosal tolerance; CD3 monoclonal antibody; NOD; Treg

1. INTRODUCTION

The number of type 1 diabetes mellitus (T1D) patients in the US has been estimated 1–1.5 million. T1D is a chronic progressive autoimmune disease that results in the destruction of insulin producing pancreatic β -cells, leading to 3–4 times increased mortality as compared to non-diabetic individuals, most likely from renal failure and coronary heart disease. Non Obese Diabetic (NOD) mice that spontaneously develop autoimmune diabetes have been extensively used to study mechanisms underlying the development of autoimmune diabetes and therapeutic approaches to prevent or cure the disease [1]. Due to the complexity of T1D and its pathogenesis, encompassing genetic, biological and environmental factors, current treatments are limited to restoring insulin availability, normally by lifelong administration of exogenous insulin and if necessary by transplantation of pancreatic islets.

Intravenous administration of CD3-specific monoclonal antibodies (mAb) has been shown to treat ongoing disease in animal models of autoimmunity such as diabetes in NOD mice [2,3] and experimental allergic encephalomyelitis (EAE), a model for multiple sclerosis [4]. Intravenous CD3-specific mAb has shown promising results in clinical trials in patients with recent onset type 1 diabetes [4,5], though phase 3 trials did not meet their primary endpoints, most likely due to changes in dosing and altered endpoints [6,7]. Although minimal, side effects did occur. Oral administration of CD3-specific mAb is an alternative therapeutic approach targeting CD3 on the surface of T cells that has the advantage of its tolerability with virtually no side effects [8]. Whereas intravenous CD3-specific mAb induced tolerance depends on depletion of pathogenic T cells while preserving regulatory T cells [9,10] oral CD3-specific mAb relies on induction of regulatory T cells [11,12]. Oral CD3-specific mAb

has demonstrated therapeutic efficacy in a number of animal models [13–15] including experimentally induced diabetes by streptozocin [16].

Since NOD mice develop spontaneous autoimmune diabetes that better resembles human type 1 diabetes and due to the availability of transgenic NOD mice expressing the human CD3 epsilon chain, allowing preclinical testing of human CD3-specific mAb, we investigated the ability of oral CD3-specific mAb to protect NOD mice from diabetes and the effect of the treatment on the immune response.

2. MATERIAL AND METHODS

2.1 Mice

NOD (purchased from Jackson Laboratory), NOD.SCID (from Charles River) and NOD-huCD3 ϵ mice (developed by the lab of Lucienne Chatenoud) were housed in a specific pathogen-free animal facility at the Harvard Institutes of Medicine according to the animal protocol guidelines of the Committee on Animals of Harvard Medical School, which also approved the experiments. Some experiments were performed at the “Institut Necker-Enfants Malades, INSERM U1151- CNRS UMR 8253” with mice that were bred and housed there according to European Directives (86/609/EEC) and institutional guidelines. The animal facility has an agreement delivered by the Prefecture de Police of Paris, France.

2.2 Antibodies and reagents

Monoclonal anti-mouse CD3 monoclonal antibodies (CD3-specific mAb; 145-2C11 or F(ab')₂ fragments of 145-2C11), anti-human CD3 mAb (OKT3) and the respective isotype controls (hamster IgG, hamster IgG F(ab')₂ fragments and mouse IgG2a clone C1.18.4) were purchased from BioXCell. The CD8 T cell epitopes GAD65_{524–543}, proinsulin (PI) B_{15–23} and the BDC2.5 mimotope_{1040–31} (Mimo) were purchased from SynBioSci.

2.3 Oral treatment

We fed 6-weeks old female NOD mice with 10 μ g CD3-specific mAb or isotype control, respectively, diluted in 2ml PBS by gavage with stainless steel feeding needles for 5 days. For the experiment represented in figure 1B F(ab')₂ fragments of 145-2C11 were used while all other experiments were performed with the entire anti-CD3 mAb 145-2C11. Depending on the experiment, we either analyzed mice on day 6, continued feeding once a week until 16 weeks of age for follow-up of diabetes development or immunized the mice 2 days after the initial CD3-specific mAb feeding with 100 μ g Mimo peptide in CFA in the ventral flanks. Mice were used for *in vitro* assays on day 10 after immunization.

2.4 Nasal treatment

For nasal treatment, we administered 10 μ g CD3-specific mAb or isotype control, respectively, diluted in 5 μ l PBS into the nostrils of 6-week old female NOD mice, using the same treatment protocol as for CD3 therapy.

2.5 Diabetes monitoring

Mice were monitored starting at 12 weeks of age or 6 weeks after adoptive transfer, respectively, once per week by measuring the glycosuria using Precision Laboratories Glucose Test Strips (Fisher Scientific). Diabetes was confirmed by testing fasting glycemia (>250 mg/dl; Bayer Contour Blood Glucose Monitoring System; Fisher Scientific).

2.6 IFN γ ELISPOT

As previously described [17], splenocytes were cultured at 2.5×10^5 /well and pLN cells (3.0×10^4 /well) were co-cultured with 2.0×10^5 irradiated (35 Gy) splenocytes/well, in presence of IL-2 (1 U/well) in ELISPOT plates (Millipore) that were coated with IFN γ capture antibody (U-CyTech Biosciences). Cells were stimulated for 20 hrs with the CD8 T cell epitopes IGRP₂₀₆₋₁₄ or PPI-B₁₅₋₂₃ (7 μ M) as compared to a negative control (a mixture of viral peptides from EBV, CMV and HIV; 7 μ M) and a positive control (CD3-specific mAb; 145 2C11; 1 μ g/ml) and processed according to the manufacturer's protocol (U-CyTech Biosciences). The air-dried plates were analysed using an AID reader (Autoimmun Diagnostika). Results are shown as spot-producing units (SPU) per 10^6 responder cells following background subtraction.

2.7 Proliferation

Single cell suspensions were prepared from spleen and pancreatic lymph nodes (pLN). To measure proliferation, cells were plated in 96-well round bottom plates (5×10^5 cells/well) in X-Vivo15 medium and stimulated with 1 μ g/ml, 0.2 μ g/ml or 0.04 μ g/ml soluble CD3-specific mAb or indicated concentrations of MIMO, GAD and Insulin peptides in culture at 37°C, 5% CO₂. After 72 hrs supernatants were removed and 1 μ Ci/well [³H]-thymidine in X-vivo15 was added. Proliferation was assessed using a 1450 Microbeta liquid scintillation counter (Perkin Elmer).

2.8 Antibodies and FACS analysis

Cells were stained in Mg²⁺ and Ca²⁺ free HBSS with 2% FCS, 0.4% EDTA (0.5 M) and 2.5% HEPES (1M) and either directly acquired or fixed in PBS containing 2.5% formaldehyde (Sigma-Aldrich, Steinheim, Germany). FoxP3 (FJK-16s) was detected by intracellular staining according to the manufacturer's instructions (eBioscience, San Diego, CA). Cells were acquired on a FACS LSRII or Fortessa (BD) and analysed using FlowJo software. Fixable viability dyes eFluor780 or eFluor506, Sytox Red, AnnexinV and Fluorochrome conjugated antibodies to mouse CD3 (145-2C11), CD4 (RM4-5), CD8a (53-6.7), GARP (YGIC86), CD62L (MEL-14), CD44 (IM7), CD25 (PC61.5), PD1 (J43), FoxP3 (FJK-16s) were purchased from eBioscience. Anti-mouse LAP (TW7-16B4) was from BioLegend.

2.9 Cytokine ELISA

Supernatants were harvested after 48 hrs of culture and the concentrations of indicated cytokines were measured by quantitative capture ELISA according to the guidelines of the manufacturer (R&D Systems).

2.10 Histology

Pancreata were fixed in AFA, embedded in paraffin, cut and stained with hematoxylin/eosin at the HMS Rodent Histopathology Core.

2.11 Adoptive transfer

We treated female, 12 weeks old, non-diabetic NOD-huCD3e mice [18] for five consecutive days with 50µg 145-2C11, OKT3 or isotype control (5 per group). On day 6 we isolated spleen cells, and FACsorted TCR+CD62L⁻ and TCR+CD62L⁺ T cells. As described in the results section we transferred 5*10⁶ spleen cells, 5*10⁵ TCR+CD62L⁻ T cells (effector T cells) or 5*10⁵ TCR+CD62L⁻ T cells together with 1*10⁶ TCR+CD62L⁺ T cells to 6 weeks old female NOD.SCID recipients that were followed-up for diabetes development for 15 weeks.

2.12 Statistical Analysis

GraphPad Prism 6.0 was used for statistical analysis. The occurrence of diabetes was plotted using the Kaplan-Meier method. Statistical comparison between the curves was performed using the logrank (Mantel-Cox) test. When appropriate, the Student's t test or one-way analysis of variance, followed by Tukey multiple comparisons was used. A p-value < 0.05 was considered statistically significant.

3. RESULTS

3.1 Mucosal CD3-specific mAb protects NOD mice from diabetes

We orally administered 10 µg CD3 specific monoclonal antibody (CD3-specific mAb; 145-2C11) to 6 weeks old female NOD mice once daily for the first 5 days and then once per week until 20 weeks of age. Diabetes incidence was significantly reduced (50% versus 87.5%; p<0.05) as compared to isotype treated mice at 25 weeks of age (Fig. 1A). Similarly, feeding F(ab')₂ fragments of CD3-specific mAb protected NOD mice from diabetes development (Fig. 1B; 50% versus 100% diabetics; p<0.05). We stained pancreata from 20 weeks old NOD mice with hematoxylin/eosin (H&E) to assess the impact of oral CD3-specific mAb (145-2C11) on infiltration of pancreatic islets. In accordance with the reduced incidence of diabetes in CD3-specific mAb (145-2C11) treated mice, pancreata from 20 weeks old CD3-specific mAb treated mice showed significantly less islet infiltration as compared to isotype treated control (48.9% ± 14.0% versus 11.6% ± 8.3%; p<0.05) while having a tendency towards less invasive infiltrates (Fig. 1C; 22.1% ± 9.1% versus 49.9% ± 15.3%). As nasal administration of CD3-specific mAb has also been shown to induce tolerance in animal models of autoimmunity [15,19], we tested if nasal CD3-specific mAb (145-2C11) prevents diabetes onset in NOD mice. Indeed, we observed a significant decrease of diabetes incidence (Fig. 1D; 37.5% versus 87.5%; p<0.05). Given that oral CD3-specific mAb has already been given to humans [20–22] we focused our investigation on the oral route of administration.

3.2 Oral CD3-specific mAb reduces T cell proliferation and increases TGF- β and IL-10 production following non-antigen specific restimulation

To investigate mechanisms underlying the protection from diabetes development conferred by oral CD3-specific mAb we performed *in vitro* and *in vivo* experiments at different time-points after CD3-specific mAb feeding. First, we analyzed proliferation and cytokine secretion of spleen cells of 7 weeks old NOD mice 24 hours after the fifth dose of CD3-specific mAb in response to *in vitro* stimulation with soluble CD3-specific mAb. Feeding of NOD mice with CD3-specific mAb decreased proliferation of splenocytes after *in vitro* restimulation ($29,873 \pm 1,717$ versus $55,475 \pm 2,346$; $n=3$; Fig. 2A)

Supernatants of restimulated splenocytes isolated from CD3-specific mAb fed mice contained elevated concentrations of TGF- β (Fig. 2B; 798.5 ± 28.5 pg/ml versus 592.0 ± 25.0 pg/ml) and IL-10 (Fig. 2C; $4,149 \pm 197$ pg/ml versus $3,119 \pm 54$ pg/ml) as compared to isotype fed mice, suggesting the induction of a pro-tolerogenic environment by feeding of CD3-specific mAb.

Staining of splenic T cells with a viability dye, AnnexinV (Fig. 2D–F) and PD-1 (Fig. 2G–H) showed that hypoproliferation of spleen cells from CD3 mAb fed mice was not due to increased cell death (Fig. 2D–F) or anergy (Fig. 2G–H) after *in vitro* restimulation with CD3-specific mAb. T cells from CD3 mAb fed mice even showed reduced cell death after 2 days of *in vitro* restimulation with CD3-specific mAb ($39.9 \pm 6.0\%$ versus $54.6 \pm 2.2\%$; $n=4$) as compared to controls.

3.3 Oral Anti-CD3 in animals immunized with a beta-cell antigen have reduced antigen-specific T cell proliferation and IL-17 and IFN γ production

As we detected hyporesponsiveness of spleen cells from CD3-specific mAb fed mice following non-antigen specific restimulation we assessed the ability of CD3-specific mAb treatment to affect a beta-cell antigen specific immune response. We circumvented the low islet antigen specific T cell response in 8 weeks old NOD mice by immunizing the animals with the beta-cell antigen BDC2.5 mimotope₁₀₄₀₋₃₁ (Mimo). We fed 6 weeks old NOD mice for five days with CD3-specific antibody or isotype control, immunized the mice two days later with Mimo in CFA and restimulated spleen and pancreatic lymph node (pLN) cells ten days after immunization. Both spleen (Fig. 3A; $3,395.0 \pm 554.2$ cpm versus $5,448.0 \pm 70.24$; $p<0.0001$) and pLN (Fig. 3B; 264 ± 27 cpm versus $2,526 \pm 297$ cpm; $p<0.001$) cells from treated mice showed markedly lower proliferation to Mimo restimulation than the control group. CD3-specific mAb feeding reduced IL-17 secretion by pLN cells (199 ± 36 pg/ml versus $1,583 \pm 140$ pg/ml) and IFN γ (Fig. 3E, F) production by both splenocytes (Fig. 3E; 7.8 ± 0.1 ng/ml versus 33.2 ± 0.8 ng/ml; $p<0.05$) and pLN cells (Fig. 3F; 24.1 ± 1.1 ng/ml versus 50.6 ± 0.1 ng/ml; $p<0.05$) after restimulation with Mimo. These results demonstrate that anti-CD3 mAb feeding skews antigen-specific T cells towards a less inflammatory phenotype.

3.4 Oral CD3-specific mAb renders T cells less diabetogenic

Having addressed early changes in the immune response after CD3-specific mAb feeding we investigated if oral CD3-specific mAb changes the profile of autoreactive T cells

at 16 weeks, an age at which many T cells infiltrate pancreatic islets. IFN γ ELISPOT demonstrated that feeding of CD3-specific antibody significantly decreased the reactivity of spleen cells (Fig. 4A; 3.5 ± 2.3 spot producing units (SPU) versus 24.3 ± 8.4 SPU; $p < 0.05$) against the CD8 epitope proinsulin (PI) B:15–23, in contrast to cells from pancreatic lymph nodes (Fig. 4B; 5.0 ± 3.1 SPU versus 7.0 ± 4.1 SPU). Reactivity of splenocytes (Fig. 4C; 50.1 ± 22.7 SPU versus 115.1 ± 54.1 SPU) and pLN cells (Fig. 4D; 44.8 ± 16.9 SPU versus 100.1 ± 32.6 SPU) to stimulation with the CD8 epitope IGRP 206–214 was unaltered. Splenocytes from CD3-specific mAb fed mice exhibited decreased proliferation after restimulation with soluble CD3-specific mAb (Fig. 5A) as compared to the control group. We also found significantly less IFN γ (Fig. 5B; 9.1 ± 1.2 ng/ml versus 26.3 ± 3.8 ng/ml; $p < 0.05$) and IL-17 (Fig. 5C; 394 ± 22 pg/ml versus 687 ± 135 pg/ml; $p < 0.05$) in response to soluble CD3-specific mAb (as shown for 1 μ g/ml).

Flow cytometric analysis of regulatory T cell markers revealed an increase of CD4+FoxP3+ in spleens (Fig. 5D; $14.5\% \pm 0.8\%$ versus $11.3 \pm 0.5\%$) but not in pancreatic lymph nodes (Fig. 5D; $10.6\% \pm 0.5\%$ versus $11.5 \pm 0.6\%$) of anti-CD3 mAb fed mice at 20 weeks of age. There no significant change of CD4+LAP+ T cells in either, spleen (Fig. 5E; $0.9 \pm 0.1\%$ versus $1.0 \pm 0.3\%$) or pancreatic lymph nodes (Fig. 5E; $1.7 \pm 0.4\%$ versus $0.9 \pm 0.4\%$) T cells.

Treatment of 12 weeks old female NOD mice expressing the human CD3 epsilon chain (NOD-huCD3 ϵ) with five doses of either anti-mouse CD3 mAb or anti-human CD3 mAb significantly protected NOD.SCID recipients from diabetes transfer as compared to isotype control fed mice (Fig. 5F). This decreased transfer of diabetes by spleen cells from CD3-specific mAb treated mice could be due to either, decreased diabetogenicity of effector T cells and/or increased regulation by regulatory T cells.

To distinguish between these possibilities, we analyzed diabetes transfer by diabetogenic T cells that are enriched in the CD62L $^-$ T cell population [23] (Fig. 5G). Whereas CD62L $^-$ T cells from isotype control treated NOD-huCD3 ϵ mice transferred diabetes to all recipients within 10 weeks after transfer, CD62L $^-$ T cells from anti-mouse CD3 mAb or anti-human CD3 mAb treated mice only transferred diabetes to 60% and 25%, respectively, of recipients within 15 weeks (Fig. 5G), suggesting that oral CD3-specific mAb decreases the diabetogenicity of T cells.

To test if oral administration of CD3 mAb increases the suppressive capacity of regulatory T cells we co-transferred CD62L $^-$ effector T cells from isotype fed NOD-huCD3 ϵ mice together with CD62L $^+$ T cells, that have been shown to preferentially inhibit diabetes development [23], from isotype or CD3-specific mAb fed mice (Fig. 5H). CD62L $^+$ T cells from isotype fed mice significantly protected from diabetes transfer by CD62L $^-$ T cells (50% versus 100% diabetogenic). CD3-specific mAb feeding further increased the protective capacity of CD62L $^+$ T cells, with none of the recipients developing diabetes within 15 weeks after transfer (Fig. 5H). We conclude that repeated feeding of CD3-specific mAb to young prediabetic NOD mice decreases the diabetogenicity of CD4+CD62L $^-$ effector T cells while increasing the regulatory function of CD62L $^+$ T cells.

4. DISCUSSION

We found that oral and nasal administration by CD3-specific mAb decreases the incidence of diabetes in NOD mice. The therapeutic effect of oral CD3-specific mAb was independent on the Fc portion as F(ab')₂ fragments were equally effective. This has previously been observed in the EAE model [11]. Although nasal CD3-specific mAb also decreased the incidence of diabetes in NOD mice, we focused on oral CD3-specific mAb because it has shown positive biological and therapeutic effects in humans [20–22]. Histology on pancreata from 20-week old NOD mice were consistent with the protective effect of CD3-specific mAb on diabetes development as CD3-specific mAb treated mice had significantly lower islet infiltration as compared to isotype controls.

We addressed the immune mechanisms associated with the CD3-specific mAb effects using several approaches. We assessed proliferation and cytokine secretion of splenic and pLN derived T cells in response to antigen specific and non-specific stimuli. Oral CD3-specific mAb reduced proliferation as well as IL-17 and IFN γ production by T cells at early time-point after treatment, after immunization with the mimotope of diabetogenic BDC2.5 T cells and at a later time point after treatment (20 weeks of age) suggesting that oral CD3-specific mAb treatment induces long lasting hyporesponsiveness of T cells. It has been previously shown that oral administration of CD3-specific mAb doesn't induce antigenic modulation, i.e. internalization of the CD3/TCR complex or depletion of activated T cells [11], contrarily to intravenously administered CD3-specific mAb [24]. Hyporesponsiveness was not a consequence of increased cell death or anergy after restimulation *in vitro* either. T cells from CD3 mAb fed mice even seemed to be more resistant to cell death, as evaluated directly after the end of anti-CD3 mAb feeding. One explanation for this long lasting hyporesponsiveness could be the induction of regulatory T cell producing the anti-inflammatory cytokines TGF- β and IL-10 that were elevated in supernatants of restimulated T cells from CD3-specific mAb treated mice.

To assess the impact of oral CD3-specific mAb on antigen-specific T cell responses we immunized CD3-specific mAb treated mice with a β -cell antigen, a mimotope of the diabetogenic T cell clone BDC2.5 (Mimo), and assessed proliferation and cytokines secretion in response to restimulation with the same antigen. We chose this approach because the frequency of antigen-specific T cells before 12–16 weeks of age is extremely low and immunization allowed us to boost responsiveness towards a chosen antigen (Mimo in this case) and to mimic the development of autoreactive T cells specific for islet antigens. Again, we detected hyporesponsiveness in CD3-specific mAb treated mice.

As Mimo is a CD4 epitope and the response against Mimo was induced by previous immunization, we addressed changes in the response of autoreactive CD8⁺ T cells by IFN γ ELISPOT against the two dominant CD8 epitopes PI B_{15–23} [25] and IGRP_{206–214} [26] at 20 weeks of age. Oral CD3-specific mAb decreased the number of splenic (but not pLN) CD8⁺ T cells responding to PI B_{15–23} and there was a trend towards a decreased response to IGRP_{206–214} in pLN. These results suggest that oral CD3-specific mAb therapy reduces the amount and/or the pathogenicity of autoreactive T cells over the long term.

Adoptive transfer of CD62L⁻ T cells, that have been shown to be enriched in diabetogenic T cells [27], from CD3-specific mAb fed mice showed that oral CD3-specific mAb decreases the diabetogenicity of T cells which is in line with decreased proliferation and secretion of IFN γ and IL-17 in vitro.

Using NOD-huCD3 ϵ mice that express both, the mouse and human CD3 ϵ chain, we found that oral administration of the anti-human CD3 mAb OKT3 induced similar functional changes as compared to the anti-mouse CD3 mAb 145-2C11. We further analyzed qualitative and quantitative changes in regulatory T cells. Adoptive cotransfer showed improved function of regulatory CD62L⁺ T cells. Our previous studies showed that CD4⁺ T cells expressing latent membrane bound TGF- β (CD4⁺LAP⁺) play an important role in the ameliorating effect of oral CD3-specific mAb in EAE [11]. FACS analysis of regulatory T cell subsets in 20 weeks old NOD mice after oral treatment with CD3-specific mAb showed an increase of CD4⁺FoxP3⁺ in spleens but not pancreatic lymph nodes and we didn't detect an increase of CD4⁺LAP⁺ T cells in either organ. The absence of an increase of CD4⁺LAP⁺ T cells may be related to alterations of regulatory T cell function in NOD mice [28,29]. Consistent with this, it has been reported that expression of the adapter protein GARP that tethers LAP membrane [31] is reduced in NOD mice [28] and the percentage of CD4⁺LAP⁺ T cells in NOD mice that we report in this article are much lower than the ones reported for other mouse strains [11,14,16]. Furthermore, IL-2 is known to increase GARP that is required for membrane bound LAP [30]. NOD mice display a qualitative diminution of IL-2 production that contributes to autoimmunity [29,31,32] and a genetic predisposing factor to development of autoimmune diabetes in humans and NOD mice is linked to IL-2/IL-2R gene polymorphisms [32].

CD3-specific mAb therapy is a promising approach for therapy of autoimmune diseases and other immune mediated disorders as it has been shown to be effective in a wide variety of autoimmune and inflammatory conditions [8]. Oral CD3-specific mAb is advantageous over intravenous administration as it stimulates natural mucosal physiologic mechanisms, is well tolerated [11,20–22] and can be given over long periods of time. Two single blind randomized placebo controlled phase 2a studies of oral OKT3 antibody in patients with treatment resistant chronic hepatitis C infection (HCV) [22] or nonalcoholic steatohepatitis (NASH) [21] reported positive effects on disease and immunological markers including an increase of regulatory T cells. Our findings suggest that oral anti-CD3 mAb does not only induce tolerance by its impact on regulatory T cells but also by modulating autoreactive T cells in a direct or indirect manner. Oral anti-CD3 may find efficacy in the treatment or prevention of type 1 diabetes.

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Highlights

1. Mucosal monoclonal CD3 antibody (CD3 mAb) protects NOD mice from diabetes
2. Oral administration of CD3 mAb decreases diabetogenicity of effector T cells
3. Oral CD3 mAb increases the number and function of regulatory T cells

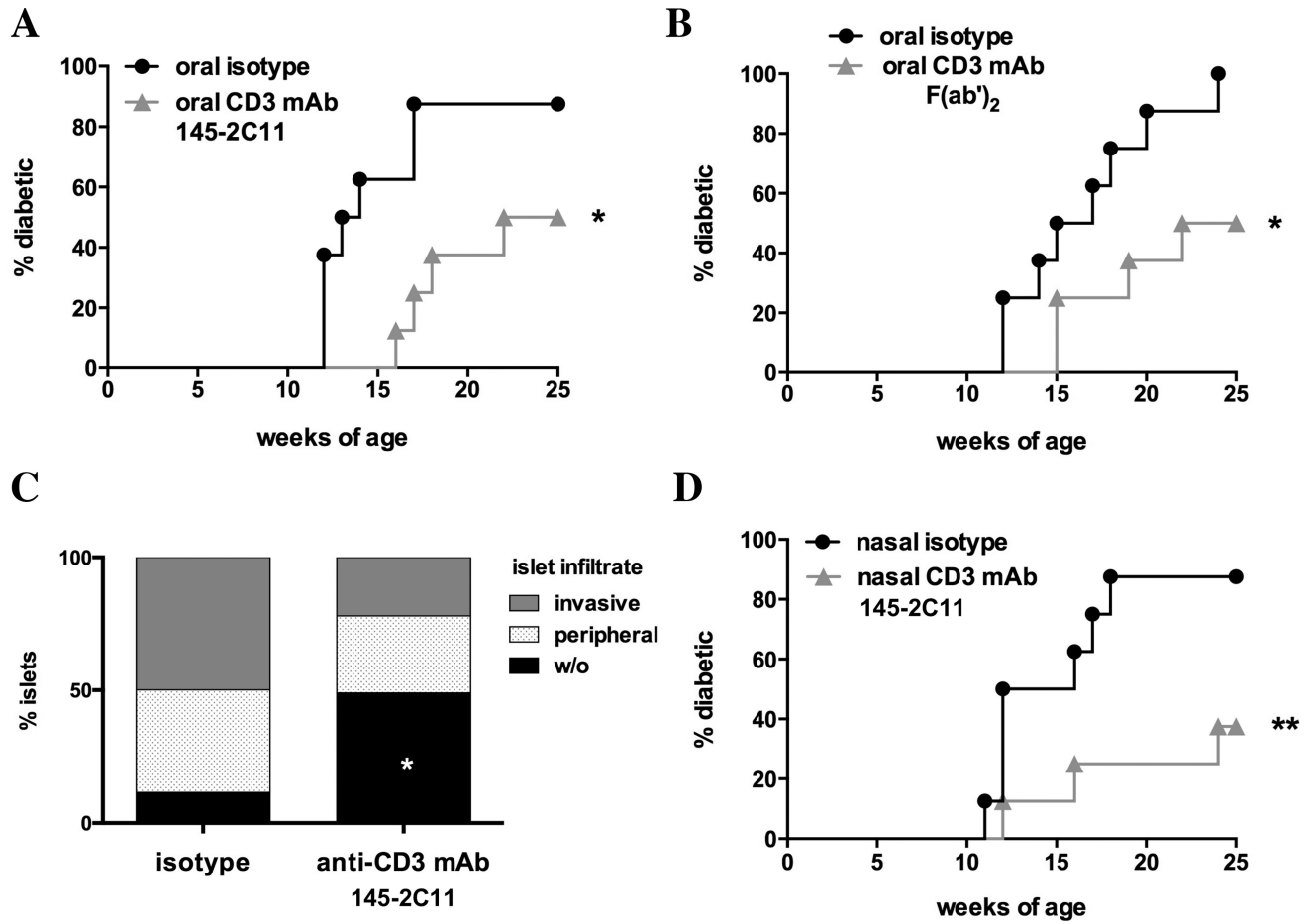


Figure 1. Diabetes incidence after oral CD3-specific mAb

A–B. Spontaneous diabetes development in NOD mice treated with oral CD3-specific mAb (A; 145-2C11; grey triangles; n=8) as compared to hamster IgG (A; black circles; n=8) or F(ab')₂ fragments of 145-2C11 (B; grey triangles; n=8) as compared to F(ab')₂ fragments of hamster IgG (B; black circles) for 5 days and then once a week (50 µg per dose) until week 20. **C.** Islet infiltration of pancreata from 20 weeks old NOD mice treated with CD3-specific mAb (145-2C11; n=8) as compared to isotype control (n=7). **D.** Diabetes incidence after treating NOD mice with nasal CD3-specific mAb (145-2C11; grey triangles; n=8) as compared to hamster IgG (black circles; n=8) using the same protocol as in A and B. Statistically significant results are indicated in the figure: *p<0.05; **p<0.01.

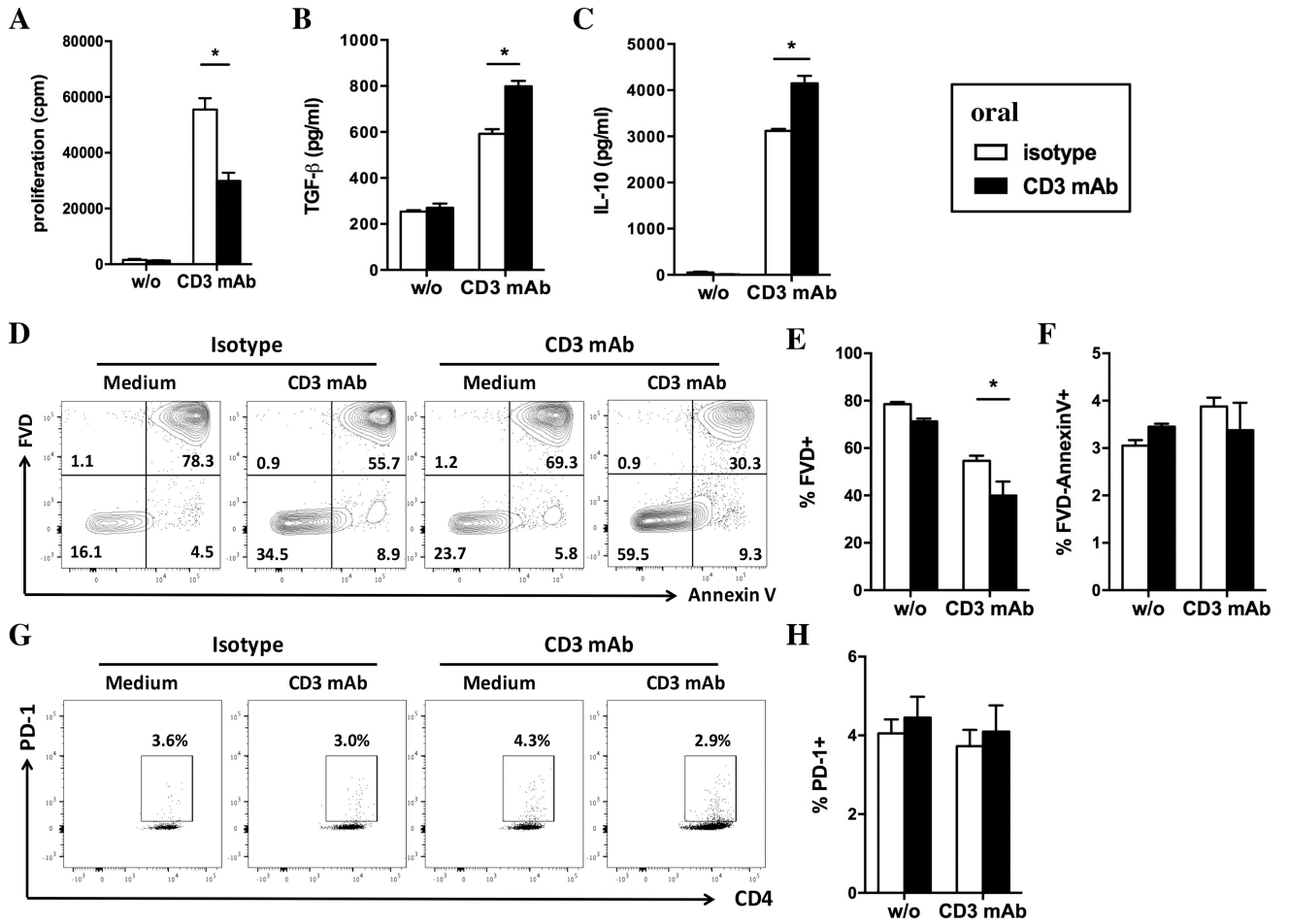


Figure 2. *In vitro* analysis of spleen cells after five days of oral CD3-specific mAb
A. Proliferation of splenocytes from CD3 mAb fed mice (black bars) as compared to isotype fed mice (white bars) after 72 hrs stimulation with of CD3-specific mAb *in vitro* (1 μg/ml).
B–C. Concentration of TGF-β (B) and IL-10 (C) in the supernatant of spleen cells from CD3 mAb fed as compared to isotype fed mice after 48 hrs stimulation with CD3-specific mAb. **D–G.** Analysis of cell death (D, E) and apoptosis (D, G) in splenic T cells from CD3 mAb fed mice as compared to isotype fed mice after 48 hrs stimulation with of CD3-specific mAb *in vitro* (gated on lymphocytes). Dead cells were detected using a fixable viability dye (FVD; D, E) and apoptotic cells were defined at FVD-AnnexinV+ (F). PD-1 staining on CD4+ T cells after 48 hrs of stimulation with CD3-specific mAb. Results are shown as mean ± SEM (n=3–4). In all panels black bars represent cells from anti-CD3 mAb fed mice whereas white bars show data from isotype control fed mice. Statistically significant results are indicated: *p<0.05, ** p<0.01, ***p<0.001.

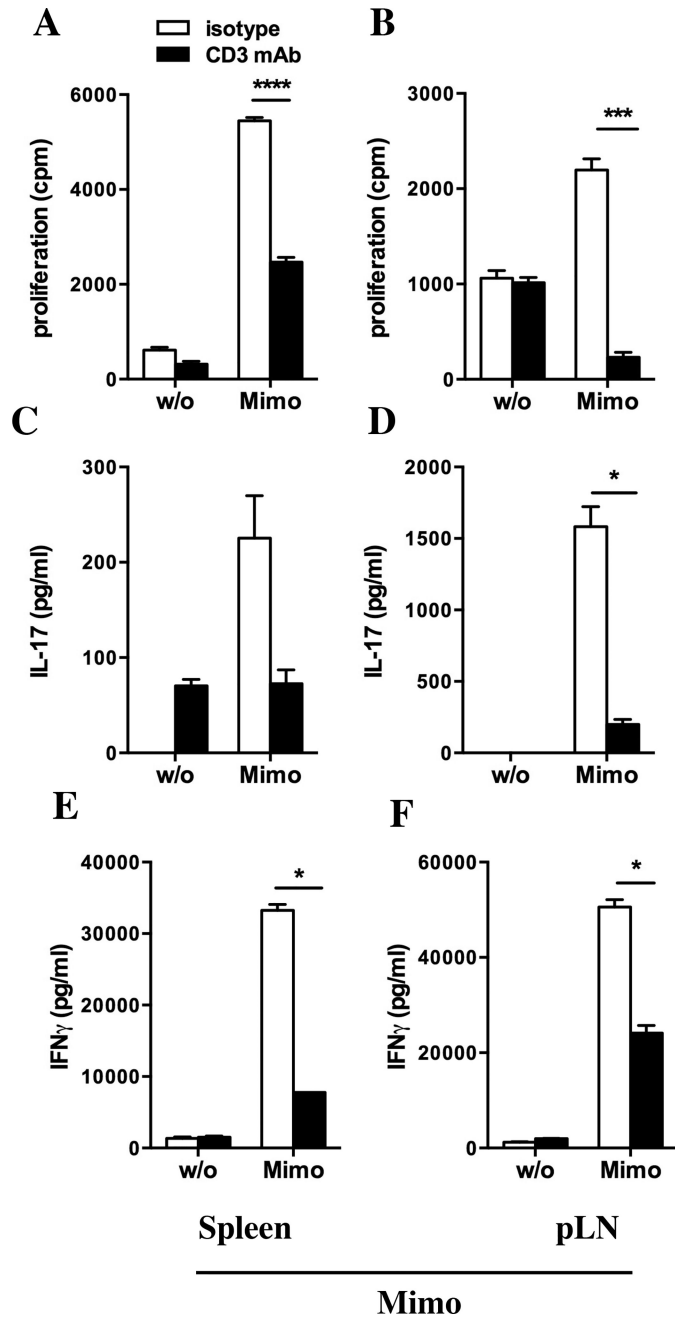


Figure 3. *In vitro* analysis of spleen and pLN cells after five days of oral CD3-specific mAb and immunization with the beta-cell antigen MIMO
A–F. Analysis of proliferation (A, B) and secretion of IL-17 (C, D) and IFN γ (E, F) of spleen cells (first column) and pancreatic lymph node (pLN) cells (second column) from anti-CD3 mAb fed (black bars) or isotype fed mice (white bars), 10 days after immunization with MIMO, in response to restimulation with MIMO (100 μ g/ml). Results are shown as mean \pm SEM (n=3). Statistically significant results are indicated: *p<0.05; ***p<0.001; ****p<0.0001.

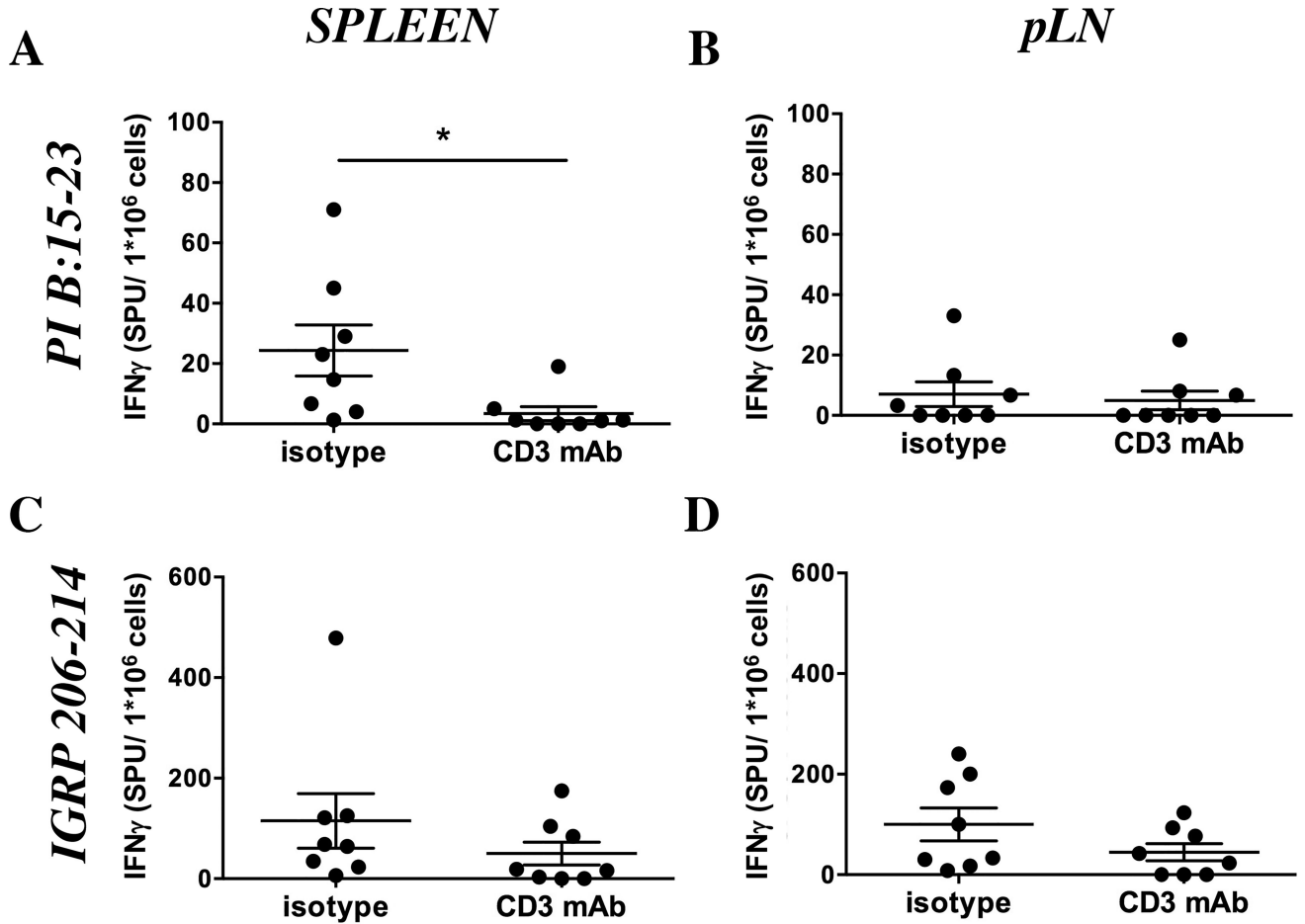


Fig 4. IFN γ ELISPOT analyzing CD8⁺ T cell responses following oral CD3-specific mAb therapy

A – D. IFN γ ELISPOT response of spleen (A, C) and pancreatic lymph node (pLN; B, D) cells from anti-CD3 mAb or isotype control fed mice in response to the CD8 epitopes PI B₁₅₋₂₃ (A, B) and IGRP₂₀₆₋₂₁₄ (C, D) in spot producing units (SPU) per 1*10⁶ cells. Graphs show average number \pm SEM of SPU per million responder cells following background subtraction (n=7–8). Results from two independent experiments were pooled. Statistically significant results are indicated: *p<0.05.

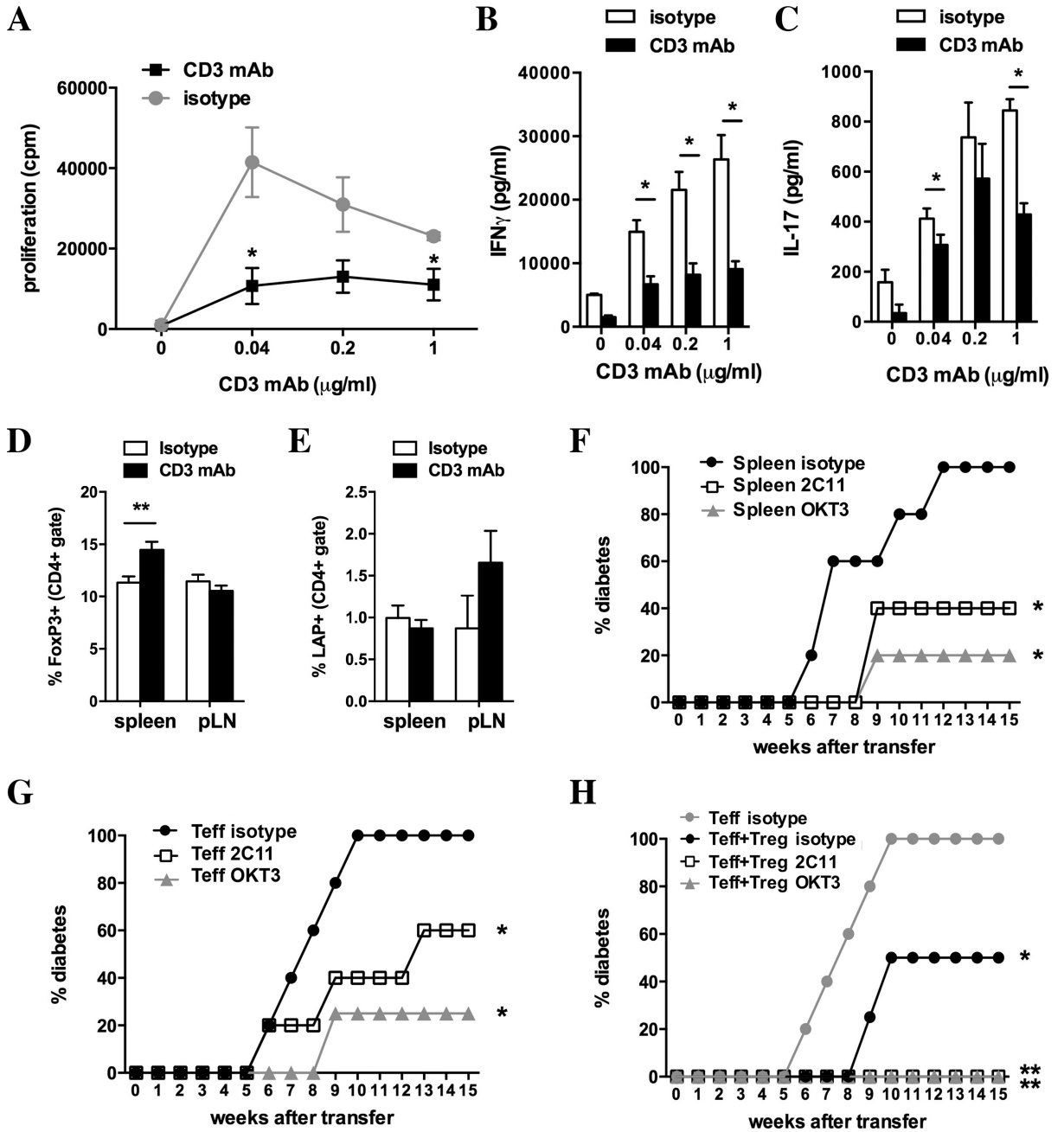


Fig 5. Analysis of effector T cells and regulatory T cells after oral CD3-specific mAb
A–C. Proliferation (A), IFN γ (B) and IL-17 (C) production of spleen cells from 20 weeks old mice that have been fed with anti-CD3 mAb (black circles/ bars) or isotype control (white circles/ bars) in response to indicated concentrations of CD3-specific mAb *in vitro*.
D–E. Percentage of CD4+FoxP3+ (D) and CD4+LAP+ (E) T cells in spleens and pancreatic lymph nodes (pLN) of anti-CD3 mAb (black bars) or isotype control (white bars) fed mice.
F–H. Diabetes incidence of NOD.SCID mice (n=4–5 per group) having received 5×10^6 spleen cells (F) or 0.5×10^6 CD62L $^-$ effector T cells alone (G) or together with CD62L $^+$ regulatory T cells (H) from 16 weeks old NOD-huCD3e mice that were fed for five days

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with the anti-mouse CD3 mAb 145-2C11 (2C11; white squares), anti-human CD3 mAb OKT3 (grey triangles; n=5) or isotype control (black or circles; n=4–5; F–H or grey circles n=5; H). Statistically significant results are indicated in the figures: *p<0.05.

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