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Supplementary Materials for

Human CD3 Transgenic Mice: Preclinical Testing of Antibodies Promoting Immune Tolerance

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SUPPLEMENTARY MATERIAL (C. Kuhn et al.)

MATERIALS AND METHODS

Figure S1

Splenic T cells from NOD or NOD-huCD3ε mice were isolated by negative selection using magnetic bead activated cell sorting (MACS) T-cell isolation kit (Miltenyi Biotech). Cells were incubated for 30min at 4°C with the indicated CD3 antibody (1µg/$1x10^6$ cells, $1x10^8$ cells/ml). After washing twice in ice-cold phosphate buffer saline (PBS) to remove unbound antibody, $1x10^6$/ml cells were incubated at 37°C to induce antigenic modulation. Modulation was stopped at different points in time by fixation for 15min with 4% paraformaldehyde (PFA). After washing cells were allowed to adhere on poly-L-lysine-coated (Sigma-Aldrich) glass slides for 1hr at room temperature. Before staining PFA was quenched with PBS-glycine. Cells were permeabilized for 20min at room temperature using PBS-0.2% saponine (Sigma-Aldrich) and staining was performed after blocking with PBS-0.1% saponine-0.5% bovine serum albumin (BSA) for 10min. Surface bound or internalized CD3 antibodies were detected by fluorochrome-coupled secondary antibodies; lysosomes were labelled in two steps using LAMP-FITC (Beckton Dickinson) and anti-FITC-Alexa488 (Molecular Probes). The slides were mounted after consecutive incubation in PBS-formaldehyde and NH_4Cl using fluoromount G. Acquisition of images was performed on a Leica confocal microscope (TCS SP5 AOBS).

Figures S2, S3 and Tables 1 and 2

Methods are described in the manuscript

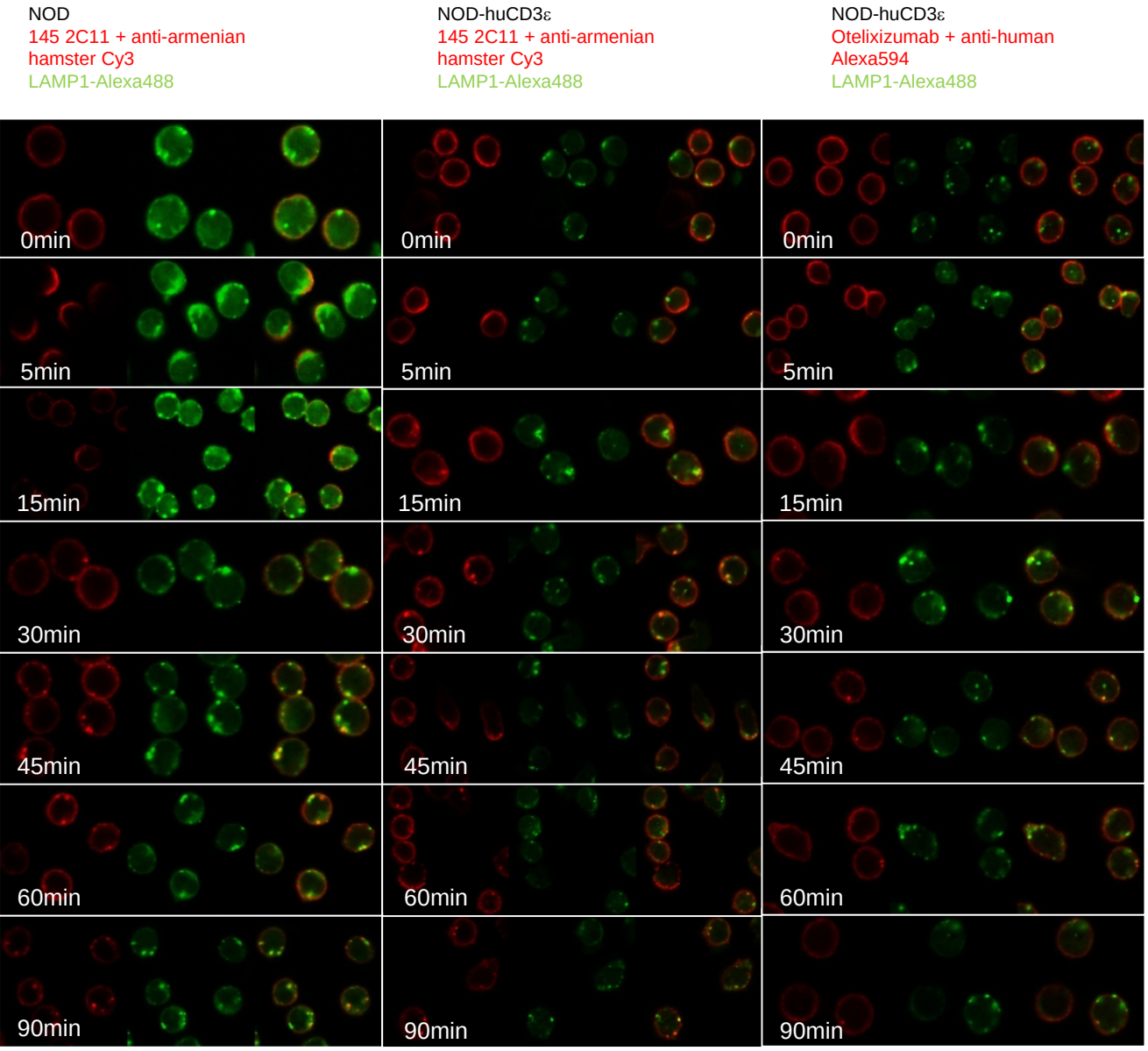
Figure S4

For Panel A : Methods are described in the manuscript

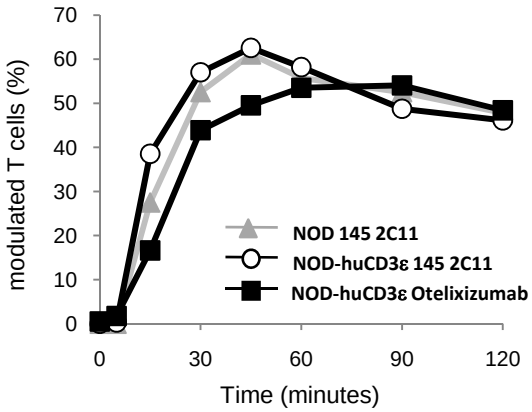
For Panel B : 6µm frozen pancreas sections were fixed in acetone for 20min at -20°C. After blocking unspecific sites for 30min with PBS-2% BSA the sections were incubated for 1hr with the primary antibodies at RT in the dark. We used primary antibodies to insulin (biotinylated D3E7; Abcam), murine CD3 (145 2C11) and FoxP3 (FJK-16s). The slides were washed in PBS, incubated for 30min with fluorochrome conjugated secondary antibodies (Streptavidin-Cy3 (Biolegend), goat-anti-armenian-hamster IgG-Cy5 (Jackson ImmunoResearch) and donkey-anti-rat-Alexa488 (Molecular Probes)) and mounted with Fluoromount G (Southern Biotech) after a final washing step. The confocal images were acquired on a Leica SP5 AOBS and analysed using ImageJ.

Figure S1 : Antigenic modulation of CD3-TCR induced in vitro by anti-mouse or anti-human CD3 antibodies.

A.



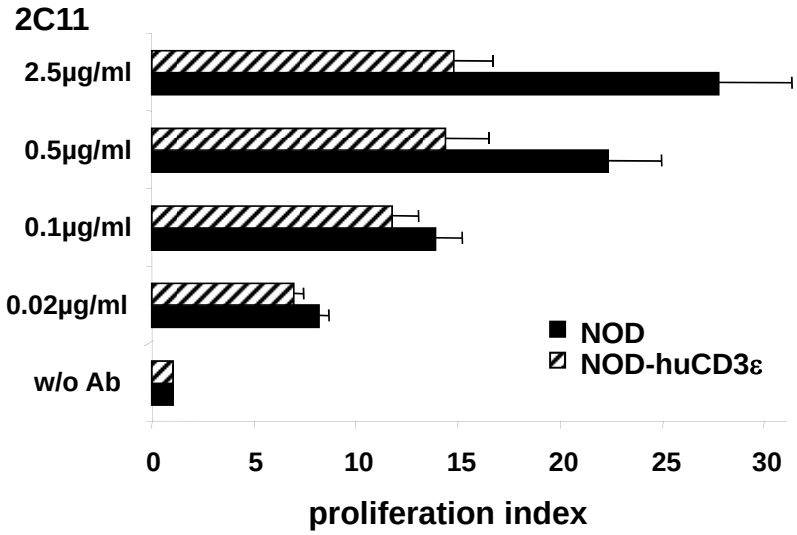
B.



LEGEND TO FIGURE S1

Antigenic modulation was assessed by confocal microscopy following in vitro incubation of T cells from wild-type NOD or NOD-huCD3ε mice with the indicated monoclonal antibodies. Representative images are shown describing kinetics of internalization and degradation (**A**) of 2C11 (anti-mouse CD3 antibody) in NOD T cells, (**B**) of 2C11 in NOD-huCD3ε T cells and (**C**) of Otelixizumab in NOD-huCD3ε T cells. (**D**) Proportion of T cells with internalized CD3 antibodies as assessed by confocal microscopy (at least 200 cells per time point have been analyzed).

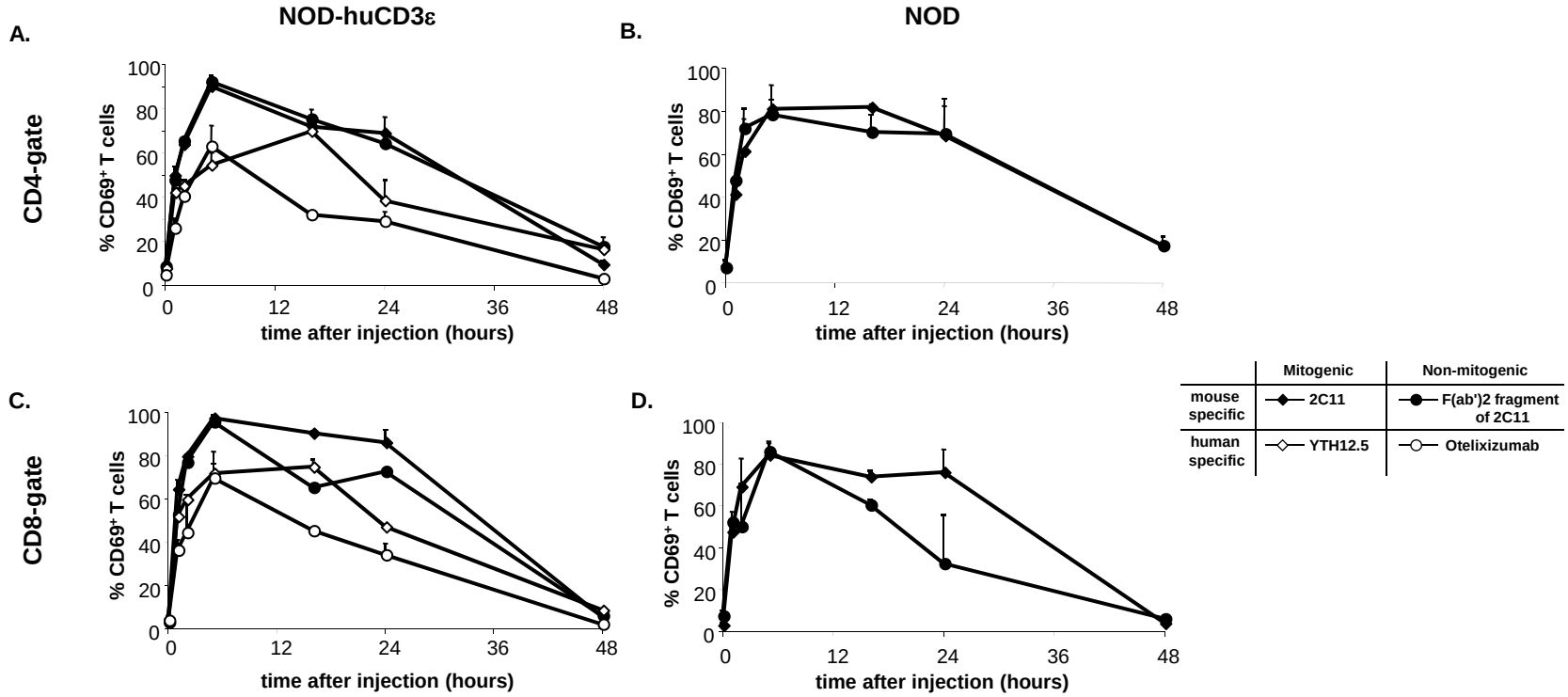
Figure S2 : Proliferative response of splenocytes from NOD and NOD-huCD3ε mice to mitogenic anti-mouse CD3 antibody.



LEGEND TO FIGURE S2

Representative data showing dose-dependent proliferation of splenocytes from NOD and NOD-huCD3ε mice to rising concentrations of 2C11 as assessed by ^{3}H-thymidine incorporation. Results are expressed as proliferation index as compared to non stimulated controls (mean ± SEM values are shown) and are representative of one out of 4 experiments conducted.

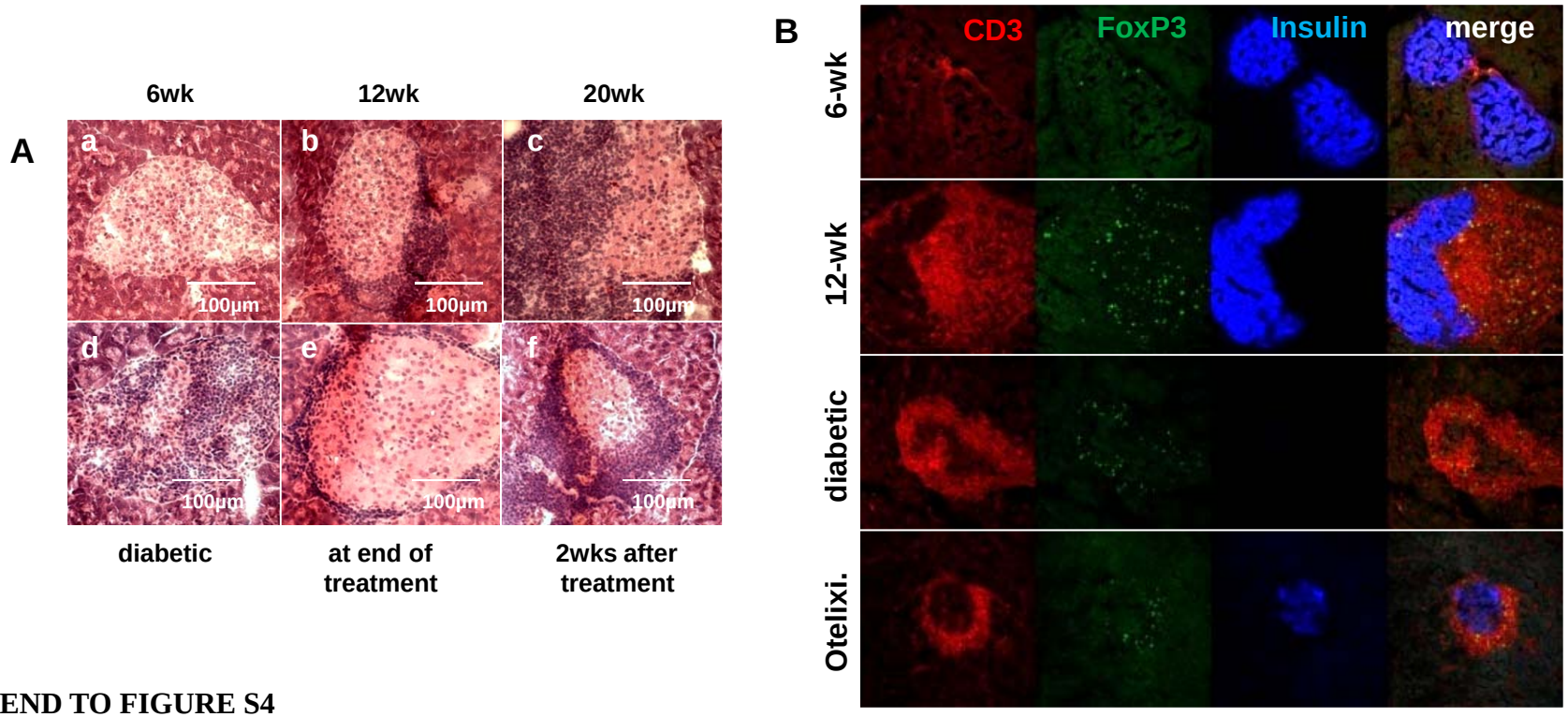
Figure S3 : Induction of T cell activation as assessed by CD69 staining after in vivo CD3 antibody administration.



LEGEND TO FIGURE S3

Kinetics of CD69 expression on $CD4^+$ and $CD8^+$ T cells from NOD or NOD-huCD3ε mice following a single injection of mitogenic or non mitogenic anti-mouse (2C11 or $F(ab)'_2$-2C11) or anti-human CD3 (YTH12.5 or Otelixizumab) antibodies (mean ± SEM values of 3 independent experiments are shown).

Figure S4 : Insulitis in untreated and CD3 antibody-treated NOD-huCD3ε mice.

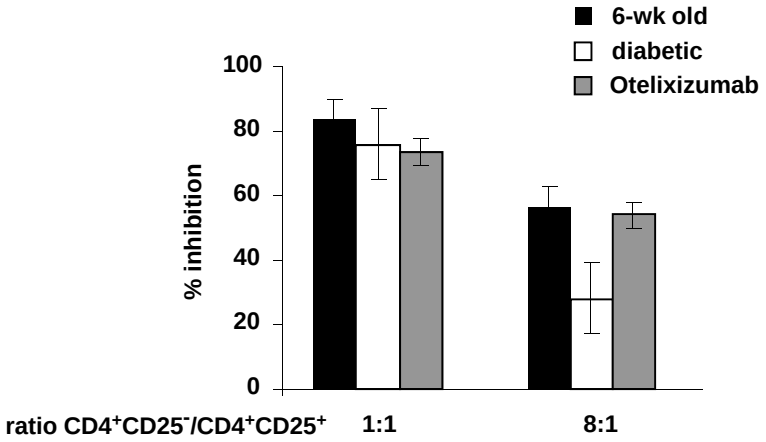


LEGEND TO FIGURE S4

Panel A Photomicrographs representing insulitis at various stages of disease development in untreated NOD-huCD3ε mice and after Otelixizumab treatment. Photos a), b), c) and d) show progression of infiltration in 6, 12, 20 week-old and overtly diabetic mice respectively. Invasive/destructive infiltration is observed at 20 weeks of age and in overtly diabetic animals. Following Otelixizumab administration insulitis disappears by the end of the treatment course (e)) and thereafter progressively reappears under the form of a peripheral infiltrate (f)).

Panel B Representative images from confocal microscopy showing staining for insulin (blue), $CD3^+$ cells (red) and $FoxP3^+$ cells (green) in frozen pancreas sections from 6-weeks old, 12-weeks old, diabetic and Otelixizumab-treated NOD-huCD3ε mice by 2 weeks after the end of treatment.

Figure S5 : Dose-dependent inhibition of $CD4^+CD25^-$ T cell proliferation by $CD4^+CD25^+$ regulatory T cells.



LEGEND TO FIGURE S5

Results from conventional co-culture assay where $CD4^+CD25^+$ T cells (Tregs) purified from 6-week old, untreated overtly diabetic or Otelixizumab-treated NOD-huCD3ε mice inhibit proliferation of autologous $CD4^+CD25^-$ T cells as assessed by ^{3}H-thymidine incorporation (mean±SEM values are shown). Different ratios of $CD4^+CD25^-$ /$CD4^+CD25^+$ were tested (1:1 and 8:1).

SUPPLEMENTARY TABLE 1. In vitro conversion of $CD4^+CD25^-$ peripheral T cells into $CD4^+CD25^+FoxP3^+$ T cells in the presence of CD3 antibodies and TGF-β

		NOD	NOD-huCD3ε
2C11 + anti-CD28	**without TGF-β**	2.6 ± 1.1 %	2.7 ± 0.4 %
	3ng/ml TGF-β	31.4 ± 6.8 %	35.5 ± 12.9 %
YTH12.5 + anti-CD28	**without TGF-β**	-	3.1 ± 0.7 %
	3ng/ml TGF-β	-	36.0 ± 3.0 %

Data represent mean ± SEM values of 3 independent experiments

SUPPLEMENTARY TABLE 2. Induction of T cell activation as assessed by CD25 staining after in vivo CD3 antibody administration.

% $CD25^+$ cells within	antibody treatment	NOD-huCD3ε	NOD
$CD4^+$ cells	**2C11**	77.6 ± 6.0%	61.8 ± 6.4%
	$F(ab')_2$ 2C11	78.9 ± 10.7%	57.1 ± 10.2%
	YTH12.5	31.6 ± 4.9 %	-
	Otelixizumab	16.7 ± 4.3 %	-
$CD8^+$ cells	**2C11**	93.1 ± 1.7%	70.2 ± 3.4%
	$F(ab')_2$ 2C11	88.3 ± 7.9%	74.8 ± 9.6%
	YTH12.5	30.1 ± 13.4%	-
	Otelixizumab	14.1 ± 9.2%	-

Data represent mean ± SEM values of 3 independent experiments; samples were recovered 5 hours following a single injection of the anti-mouse or anti-human CD3 antibodies