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C-type lectin-like CD161 is not a co-signalling receptor in gluten-reactive CD4+ T cells

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List of abbreviations

APC antigen presenting cell

CeD coeliac disease

cpm counts per minute

EBV Epstein-Barr virus

FCS fetal calf serum

FMO fluorescence minus one

HLA human leukocyte antigen

HS human serum

LLT1 Lectin-Like Transcript 1

LPMC lamina propria mononuclear cells

mAb monoclonal antibody

NK Natural Killer cell

PBMC peripheral blood mononuclear cells

SEM standard error of the mean

TCC T-cell clone

TCR T-cell receptor

TG2 transglutaminase 2

UCeD untreated coeliac disease

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Abstract

Surface C-type lectin-like CD161, a class II transmembrane protein, is a surface receptor expressed by NK cells and T cells. In coeliac disease, CD161 was expressed more frequently on gluten-reactive CD4+ T cells compared to other memory CD4+ T cells isolated from the same tissue compartment. CD161 is a putative co-signalling molecule that was proposed to act as co-stimulatory receptor in the context of signalling through TCR, but contradicting results were published. In order to understand the role of CD161 in gluten-reactive CD4+ T cells, we combined T-cell stimulation assays or T-cell proliferation assays with ligation of CD161 and intracellular cytokine staining. We found that CD161 ligation provided neither co-stimulatory nor co-inhibitory signals to modulate proliferation and IFN-γ or IL-21 production by gluten-reactive CD4+ T-cell clones. Thus, we suggest that CD161 does not function as a co-signalling receptor in context of gluten-reactive CD4+ T cells.

Keywords

coeliac disease, gluten-reactive CD4+ T cells, CD161

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Conflict of interest

The authors declare that they have no competing interests.

Introduction

Coeliac disease (CeD) is an intestinal disorder caused by inappropriate immune response to gluten proteins of wheat, rye and barley. The disease affects small intestinal mucosa and leads to structural alterations manifested by villous atrophy and crypt hyperplasia. Lifelong adherence to gluten-free diet is the primary treatment for CeD. This treatment will gradually induce histological normalization of disease-related changes in the gut.[1] CeD affects 1-2% of the Western population and develops exclusively in genetically predisposed individuals. The majority of CeD patients carry HLA-DQ2.5, the remaining are either HLA-DQ2.2 or HLA-DQ8 positive.[2]

Adaptive immune responses in CeD are driven by gluten-reactive CD4+ T cells that respond to tissue transglutaminase 2 (TG2)-deamidated gluten peptides presented by HLA-DQ2.5, HLA-DQ2.2 or HLA-DQ8 molecules. Gluten-reactive CD4+ T cells can be readily isolated from blood and intestine from CeD patients by use of HLA-DQ:gluten tetramers. In HLA-DQ2.5+ coeliac individuals, T-cell reactivity is predominantly directed towards immunodominant epitopes contained within α- and ω-gliadin proteins.[3] Circulating gluten-reactive CD4+ T cells are gut-homing effector memory cells[4], initially classified as Th1 cells producing high amounts of IFN-γ[5]. Some gluten-reactive CD4+ T cells also produced IL-21[6], a cytokine important for T cell-dependent B cell responses[7]. Recently it was demonstrated that gluten-reactive CD4+ T cells bear features of both Th1 and Tfh cells and that they are almost uniformly positive for CD161.[8] Moreover, single-cell RNA sequencing of peripheral blood CD4+ T cells from untreated CeD (UCeD) individual revealed, that *KLRB1* (encoding CD161) was significantly up-regulated in most of gluten-reactive CD4+ T cells as compared to control CD4+ T cells (Yao

et al., manuscript submitted), further supporting the need for functional analysis of CD161 receptor in this subset of CD4+ T cells.

CD161 is a C-type lectin-like type II transmembrane protein that was initially found on natural killer (NK) cells and different subsets of peripheral blood T lymphocytes inclusive of CD4, CD8, TCRαβ and TCRγδ cells.[9] CD4+CD161+ T cells in peripheral blood were mostly memory CD45RO+ cells comprising both central and effector memory cells.[10,11] CD4+CD161+ T cells were also abundant in the gut and located predominantly in the lamina propria compartment of colon and duodenum.[12] In T cells, CD161 is widely regarded as co-signalling molecule.[13] However, opposing studies have been published, reporting results where CD161 acted either as co-stimulatory receptor[14-19], co-inhibitory receptor[20,21] or failed to show any co-signalling properties[9,20,21]. Lectin-Like Transcript 1 (LLT1), another C-type lectin-like protein, was recognized as a ligand for CD161.[15,22] LLT1 can be found on activated lymphocytes including T cells, B cells, NK cells, monocytes and dendritic cells.[23]

Here, we have analysed gluten-reactive CD4+ T cells from UCeD patient for the surface expression of CD161. Moreover, we utilized gluten-reactive CD4+ T-cell clones (hereafter referred to as T-cell clones) generated from small intestinal biopsies of treated CeD patients to address co-signalling properties of CD161. We found that ligation of this receptor neither augmented nor inhibited TCR-mediated T cell activation assessed by either cytokine secretion or cell proliferation. Therefore, we suggest that CD161 is not a co-signalling molecule in the context of gluten-reactive CD4+ T cells in CeD.

Materials and methods

Study subjects

The study participants, including one UCeD patient (CD1982) and four treated CeD patients (CD387, CD411, CD544, CD548) that donated biological material for the generation of T-cell clones had undergone a biopsy-confirmed diagnosis according to guidelines[24]. The blood and biopsy samples were collected at Oslo University Hospital during clinical assessment.

Human biological material and tetramer staining

UCeD patient CD1982 donated 100 mL of blood and 12 duodenal biopsy samples. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation with Lymphoprep (Stemcell Technologies). Duodenal biopsies were collected into ice-cold RPMI-1640 (Sigma) and treated for 2 x 10 minutes with 2 mM EDTA in 2% fetal calf serum (FCS) in

PBS at 37ºC to remove epithelial cell layer. Samples were then digested for 40 minutes with collagenase (1 mg/mL) in 2% FCS in PBS at 37ºC. Finally, samples were homogenized using a syringe and 1.2 mm needle, then filtered through 40 μM cell strainer to obtain lamina propria mononuclear cells (LPMC).

Tetramers were prepared and used as previously described.[4,25,26] Briefly, four HLA-DQ:gluten tetramers, where each HLA-DQ molecule presented one of the gliadin epitopes: DQ2.5-glia-α1, DQ2.5-glia-α2, DQ2.5-glia-ω1 or DQ2.5-glia-ω2, were multimerized on PE-labelled streptavidin (ProZyme). Cells were incubated with the mix of four HLA-DQ2.5 tetramers for 40 minutes at room temperature, in the dark. Subsequently, PBMC samples were subjected to enrichment of tetramer-stained cells as described elsewhere[4] and stained for surface markers: CD62L-PerCP/Cy5.5 (clone DREG-56), CD14-Pacific Blue (clone M5E2), CD19-Pacific Blue (clone HIB19), CD56-Pacific Blue (clone MEM-188), integrin-β7-APC (clone FIB504) and CD161-Alexa Fluor 488 (clone HP-3G10) (all from BioLegend); CD11c-Horizon V450 (clone B-Ly6), CD4-APC-H7 (clone SK3) (all from BD Biosciences); CD45RA-PE-Cy7 (clone HI100) and CD3-eVolve605 (clone OKT3) (both from eBioscience). Pacific Blue-labelled plus Horizon V450-labelled antibodies together with LIVE/DEAD cell viability marker fixable violet stain (Invitrogen) were used to exclude non-T cells and dead cells (channel "dump"). Live, single tetramer+ or tetramer- cells were CD4+ effector memory T cells with phenotype CD3+, CD11c-, CD14-, CD19-, CD56-, CD8-, CD4+, CD45RA- and CD62L- (Supplementary Figure 1A). Flow cytometry was performed on FACSAria II (BD Biosciences) at the Flow Cytometry Core Facility at Oslo University Hospital.

Gluten-reactive CD4+ T-cell clones

Four T-cell clones were previously generated from small intestinal mucosal specimens of treated CeD patients as described elsewhere[27]. The T-cell clones were expanded without the antigen and tested for specificity before cryopreservation. All T-cell clones were reactive to deamidated gluten antigen and were either HLA-DQ2.5-restricted (TCC387.3 and TCC411.3.39) or HLA-DQ8-restricted (TCC544.1.3.1 and TCC548.1.8.5). After defrosting, cells were centrifuged and resuspended in pre-heated 10% human serum (HS) in RPMI-1640 supplemented with β-mercaptoethanol and penicillin/streptomycin. Cells were immediately utilized for T-cell stimulation, T-cell proliferation and CD161 competition assays.

T-cell stimulation assays

Antigen-independent stimulation assay was modified after Aldemir and colleagues[15] and Fergusson and colleagues[18]. The following antibodies were used: anti-CD3 (clone OKT3), anti-

CD28 (clone CD28.2), anti-CD161 (clone HP-3G10) and mouse IgG1 isotype control (clone MG1-45) (all from BioLegend), anti-CD161 biotin (clone 191B8; Miltenyi Biotec) and mouse IgG2a biotin isotype control (clone eBM2a; eBioscience). Flat-bottom 48-well plates were coated with anti-CD3 (1 μg/mL) overnight at 4ºC. Before experiment, the plates were washed twice with PBS. T-cell clones (100 000 cells/well) supplemented with soluble anti-CD28 (5 μg/mL) and soluble anti-CD161 (clone 191B8 or HP-3G10) or appropriate isotype control (IgG2a or IgG1 respectively) were added to the wells and cultured for 4 hrs and 6 hrs at 37ºC. Unless stated otherwise, anti-CD161 or matching isotype control were added to the T-cell suspension at concentration of 5 μg/mL. Alternatively, T-cell clones were supplemented with soluble anti-CD28 (5 μg/mL) and 100 000 cells/well mixed with anti-CD161 (clone 191B8) or IgG2a isotype control loaded on anti-biotin MACSiBead particles prepared accordingly to manufacturer's instructions (Miltenyi Biotec). For antigen-dependent stimulation assay, HLA-DQ8-homozygous Epstein-Barr virus (EBV)-transformed lymphoblastoid B cells (BM92) were irradiated at 75 Gy and 75 000 cells/well were incubated with peptide P1584 containing the DQ8-glia-γ1b epitope (FPEQPQQPYPEQ; 10 μM) in U-bottom 96-well plate overnight at 37ºC. T-cell suspension was supplemented with soluble anti-CD161 (clone HP-3G10; 5 μg/mL) or IgG1 isotype control (5 μg/mL) and 50 000 cells were added to each well. Cells were stimulated for 4 hrs and 6 hrs at 37ºC. In each case, Brefeldin A was added for the last 4 hrs of stimulation accordingly to manufacturer's instructions (eBioscience).

Intracellular cytokine staining and CD161 competition assay

T-cell clones were stimulated as described above and collected. The following antibodies were used for staining of surface markers: CD161-Alexa Fluor 488 (clone HP-3G10) and CD4-PerCP/Cy5.5 (clone SK3) (both from BioLegend). Cells were fixed in 4% paraformaldehyde for overnight storage at 4ºC. Subsequently, cells were permeabilized in Perm/Wash Buffer (BD Biosciences) and stained for intracellular cytokines with following antibodies: IFN-γ-APC (clone 4S.B3) (BioLegend) and IL-21-PE (clone eBio3A3-N2) (eBioscience). Flow cytometry was performed on FACSCalibur (BD Biosciences).

EBV-transformed lymphoblastoid B cells were collected and stained with following antibodies: CD19-Pacific Blue (clone HIB19) and CD161-Alexa Fluor 488 (clone HP-3G10) (both from BioLegend), and LLT1-PE (clone LLT1) (402659) (R&D Systems). Flow cytometry was performed on Attune NxT Flow Cytometer (Thermo Fisher Scientific).

T-cell clones for CD161 competition assay were fixed in 4% paraformaldehyde. Subsequently, soluble anti-CD161 (clone 191B8 or HP-3G10) or matching isotype control (IgG2a or IgG1 respectively) were added to the T-cell suspension at concentration of 5 μg/mL and incubated

for 30 minutes. Cells were thoroughly washed and stained for surface markers with following antibodies: CD161-Alexa Fluor 488 (clone HP-3G10) and CD4-PerCP/Cy5.5 (clone SK3) (both from BioLegend). Flow cytometry was performed on Attune NxT Flow Cytometer (Thermo Fisher Scientific).

All flow cytometry data were analysed using FlowJo software (FlowJo LLC).

T-cell proliferation assays

Antigen-presentation-cell (APC)-free proliferation assay was performed as described elsewhere[28] with slight modifications. The following antibodies were used: anti-CD3 (clone OKT3), anti-CD28 (clone CD28.2), anti-CD161 (clone HP-3G10) and mouse IgG1 isotype control (clone MG1-45) (all from BioLegend). Flat-bottom 96-well plates were coated with anti-CD3 overnight at 4°C. T-cell suspension was supplemented with soluble anti-CD28 and soluble anti-CD161 (5 μg/mL) or IgG1 isotype control (5 μg/mL) and 50 000 T cells were added per well. Concentration of anti-CD3 and anti-CD28 was indicated in the Results section. Each condition was performed in triplicates. APC-based proliferation assay was performed as presented elsewhere[27]. The following antibodies were utilized: anti-CD161 (clone HP-3G10) or mouse IgG1 isotype control (clone MG1-45) (all from BioLegend). EBV-transformed lymphoblastoid B cells were prepared as described above. Concentration of peptide was indicated in the Results section. T-cell suspension was supplemented with soluble anti-CD161 (5 μg/mL) or IgG1 isotype control (5 μg/mL) and 50 000 cells/well were added to pre-prepared EBV-transformed lymphoblastoid B cells. Each condition was performed in triplicates.

In both assays, samples were incubated for 72 hrs at 37°C. 20 μL (1 μCi) of 3H-thymidine (Montebello Diagnostics) was added to each well during the last 24 hrs of stimulation. 3H-thymidine incorporation was measured in β-scintillation counter as counts per minute (cpm). Mean values ± SEM were presented.

Results

Gluten-reactive CD4+ T cells express CD161

Gluten-reactive CD4+ T cells from peripheral blood and biopsy specimen of one UCeD patient (CD1982) were stained with HLA-DQ2.5 tetramers representing four immunodominant gluten epitopes (DQ2.5-glia-α1, DQ2.5-glia-α2, DQ2.5-glia-ω1 and DQ2.5-glia-ω2). Tetramer+ cells and control tetramer- cells from blood and gut were effector memory cells positive for CD161. The frequency of cells expressing CD161 was higher on tetramer+ cells than tetramer- cells

(Figure 1A and Supplementary Figure 1A). Next, we inspected T-cell clones for surface CD161 and found a high proportion of CD161 positive cells (Figure 1B). The T-cell clones had been generated from small intestinal specimens obtained from treated CeD patients and were either HLA-DQ2.5-restricted (TCC387.3 and TCC411.3.39) or HLA-DQ8-restricted (TCC544.1.3.1 and TCC548.1.8.5).

Surface CD161 is downregulated when bound by anti-CD161 monoclonal antibodies

In order to examine the functional role of CD161 on gluten-reactive CD4+ T cells, we first measured surface CD161 expression following ligation by anti-CD161 monoclonal antibodies (mAbs). For this purpose, T-cell clones were stimulated for 4 hrs and 6 hrs with plate-bound anti-CD3 and soluble anti-CD28 together with anti-CD161 or isotype control. Two anti-CD161 clones and two types of ligation were utilized. Anti-CD161 or isotype control were either coated on MACSiBead particles (clone 191B8) or used in a soluble version (clone 191B8 or HP-3G10). We observed a clear downregulation of surface of CD161 when ligated by anti-CD161 mAbs (Figure 2). Downregulation was the strongest when anti-CD161 mAbs were added directly to T-cell suspension in a soluble form and saturating concentration of 5 μg/mL, irrespective of anti-CD161 clone. In comparison, surface CD161 downregulation was much lower when anti-CD161 mAbs were immobilized on MACSiBead particles (Figure 2). Having established that surface CD161 downregulation was dependent on the type of CD161 ligation, we next sought to understand if there is any difference in downregulation capacity between soluble anti-CD161 191B8 and HP-3G10 clones. For this purpose, two T-cell clones were stimulated like above with dose titration of anti-CD161 (0.2 – 1 – 5 μg/mL). Surface CD161 downregulation was dose-dependent with anti-CD161 clone HP-3G10 being more effective than clone 191B8 when utilized at low concentration (0.2 μg/mL). In comparison, there was little or no difference between anti-CD161 clones when added at medium (1 μg/mL) or at saturating (5 μg/mL) concentrations, where the CD161 surface expression was reduced by >90% (Supplementary Figure 2A and 2B). To assess epitope competition between the unlabelled and fluorescence-labelled anti-CD161 mAbs we performed CD161 competition assay with fixed cells of T-cell clones. We observed modest decrease of surface CD161 that was stronger when the combination of anti-CD161 mAbs of the same clone were used (Supplementary Figure 2C). This demonstrates that the near-complete reduction of surface CD161 following ligation with anti-CD161 mAbs was not only caused by epitope competition between unlabelled and fluorescence-labelled anti-CD161 mAbs, but reflects biological downregulation supported by published data where CD161 surface downregulation was observed after co-incubation with LLT1-expressing cells[18]. Overall, the differences in the pattern of downregulation suggested

that the quality of CD161 ligation might be important for the functional outcome of CD161-mediated co-signalling.

Ligation of CD161 on gluten-reactive CD4+ T cells does not influence IFN-γ or IL-21 production

We next sought to understand whether observed differences in CD161 ligation were translated into cellular activation assessed by cytokine secretion. T-cell clones were stimulated for 4 hrs and 6 hrs with plate-bound anti-CD3 and soluble anti-CD28. Various methods were utilized to ligate CD161 receptor. Anti-CD161 or appropriate isotype control was added either in a soluble form or coated on MACSiBead particles (Figure 3A). Intracellular staining revealed that IFN-γ production was not influenced by ligation of CD161 irrespective of the used method. The magnitude of IFN-γ production was T-cell clone-dependent and increased over time (Figure 3A). Out of all tested T-cell clones, only T-cell clone TCC387.3 produced IL-21. However, ligation of CD161 did not affect IL-21 production by neither soluble nor MACSiBead-coated anti-CD161 mAbs (Supplementary Figure 3A). Furthermore, one T-cell clone TCC544.1.3.1 was stimulated for 4 hrs and 6 hrs with corresponding gluten peptide presented by EBV-transformed lymphoblastoid B cells used as APCs. Simultaneous ligation of CD161 with soluble anti-CD161 (clone HP-3G10) did not affect IFN-γ production (Figure 3B). The EBV-transformed lymphoblastoid B-cell line used in that assay was negative for LLT1 and CD161 (Supplementary Figure 3B).

Ligation of CD161 does not increase proliferation of CD4+ gluten-reactive T cells

Finally, we investigated the impact of CD161 ligation on cell proliferation. Two assays were utilized. In APC-free assay, cells were activated by increasing concentrations of plate-bound anti-CD3 and soluble anti-CD28 in the presence of soluble anti-CD161 (clone HP-3G10) or isotype control. In APC-based assay, T cells were activated by EBV-transformed lymphoblastoid B cells presenting gluten peptides. Anti-CD161 (clone HP-3G10) or isotype control were added in a soluble form. Two DQ8-restricted T-cell clones were used. Ligation of CD161 demonstrated slight but inconsistent effect in T-cell clone TCC544.1.3.1 (Figure 4A and 4B), whereas no effect was observed on the proliferation of T-cell clone TCC548.1.8.5 (Figure 4A). We did not observe proliferation triggered by CD161 in the absence of gluten peptides.

Discussion

Gluten-reactive CD4+ T cells are in the centre of adaptive immune responses in CeD. Although this subset has been extensively studied, little is known about the role of CD161 in that context. In order to understand it, we have directly visualized gluten-reactive CD4+ T cells from blood and gut of UCeD patient. We found that when compared to control CD4+ T cells, a higher proportion of gluten-reactive CD4+ T cells expressed CD161. Despite small sample size, owing to the limited availability of voluntary blood and biopsy donations, the presented *ex vivo* analysis is in concordance with recent mass cytometry staining of gluten-reactive CD4+ T cells[8]. Therefore, it might be considered as representative sample reflecting the trend towards increased CD161 surface expression on gluten-reactive CD4+ T cells. To investigate the functional role of CD161 in this T cell subset, we have utilized *in vitro* expanded T-cell clones generated from small intestinal lesion of treated CeD patients.

The exact function of CD161 receptor is unknown. In NK cells, CD161 is generally regarded as an autonomous inhibitory receptor.[9,15,20,22,29] In T cells, however, CD161 ligation alone was insufficient to elicit any cellular response. It was dependent on concurrent engagement of the TCR[14,16] and in some reports CD161 was found to operate as a co-stimulatory receptor to increase cytokine secretion and cell proliferation[14-19]. However, contradicting studies were published demonstrating that, in certain cases, CD161 acted as co-inhibitory molecule[20,21] or was unable to provide any co-signalling[9,20,21]. Inconsistencies in the literature suggest that CD161 might act in lineage-specific and context-specific manner. For instance, Aldemir and colleagues showed that ligation of CD161 enhanced IFN-γ production by polyclonal CD3+ T cells.[15] On the other hand, Rosen and colleagues demonstrated that ligation of CD161 had no effect on cytokine production by CD4+ T cells and reduced TNF-α secretion by CD8+ T cells.[20] Several different clones of anti-CD161 mAbs were used, likely contributing to the discrepancies between the reported findings. Therefore, we have chosen two widely used anti-CD161 clones, 191B8 and HP-3G10. Both clones have provided strong co-stimulatory effects[14,15,18,19,21] and were shown to elicit similar cellular response[21].

Ligation of CD161 by corresponding anti-CD161 mAbs caused downregulation of its surface expression. This process was rapid following interaction with anti-CD161 mAbs in a soluble form, irrespective of anti-CD161 clone. Furthermore, CD161 surface downregulation as a result of ligation with soluble anti-CD161 mAbs was dose-dependent and both anti-CD161 clones displayed similar pattern when used in saturating concentration. In contrast, downregulation was much weaker following ligation with anti-CD161 mAbs loaded on MACSiBead particles. This was partially in line with results reported by Fergusson and colleagues, where anti-CD161 clone 191B8 coated on MACSiBead particles caused gradual

and time-dependent downregulation which never completely abrogated surface expression of CD161.[18] It is possible, that anti-CD161 mAbs used for CD161 ligation in T-cell stimulation assay could block subsequent surface staining, especially in the situation where the same anti-CD161 clone HP-3G10 was utilized for ligation and staining. Consequently, what was observed as surface downregulation might in fact have occurred due to competition between fluorescent-labelled and unlabelled anti-CD161 mAbs. However, CD161 surface downregulation was also observed when a different clone of anti-CD161 mAb (clone 191B8) was used in T-cell stimulation assay. Furthermore, pre-incubation with unlabelled anti-CD161 mAbs resulted in modest decrease of surface CD161 on fixed cells, suggesting that surface staining with fluorescent-labelled anti-CD161 mAbs was only partially hindered due to epitope competition. Lastly, it was demonstrated that the pattern of CD161 surface downregulation following interaction with its biological ligand, LLT1, was similar to that elicited by anti-CD161 mAbs. After removing LLT1, surface CD161 gradually increased in a time-dependent fashion.[18] Collectively these findings indicate that the reduction in surface CD161 staining reflects downregulation rather than steric hindrance.

Downregulation of surface CD161 was not correlated with co-stimulatory or co-inhibitory signals because we observed no alterations in either cytokine secretion or cell proliferation. The magnitude of IFN-γ secretion in antigen-independent stimulation assays was T-cell clone-dependent with T-cell clone TCC544.1.3.1 being the most potent one. Unlike previously published[14,16], ligation of CD161 had no effect on cell proliferation during APC-free assay in any of T-cell clones tested. To imitate biological conditions in which gluten-reactive CD4+ T cells encounter gluten peptides, we utilized EBV-transformed lymphoblastoid B cells to serve as APCs. Simultaneous ligation of CD161 during antigen-dependent stimulation or APC-based proliferation assay had negligible impact on the final outcome. Finally, direct comparison between different assays performed with the same T-cell clone TCC544.1.3.1, including antigen-independent and antigen-dependent stimulation or APC-free and APC-based proliferation, revealed almost similar pattern with minor or no differences in cytokine secretion or proliferation between CD161 samples and Isotype samples. Similar indifferent response was observed for other T-cell clones during antigen-independent stimulation and APC-free proliferation assays. Taken together, results presented in this study indicate that CD161 is neither co-stimulatory nor co-inhibitory receptor in the context of gluten-reactive CD4+ T cells in CeD.

One potential limitation to this study is that not all clones of anti-CD161 mAb were utilized. However, clones B199, DX1 and DX12 were previously shown to be less effective in downregulating CD161 or triggering cellular response than clones 191B8 and HP-3G10.[14,18,20]

In conclusion, we have evaluated T cell response by intracellular staining for IFN-γ and IL-21 or by measuring proliferation, and found that ligation of CD161 with anti-CD161 mAbs did not provide co-stimulatory nor co-inhibitory signals in the context of TCR engagement. Despite lack of co-signalling, it is possible that CD161 plays different role that should be investigated in future experiments. Although never formally shown, it was speculated, that CD161-LLT1 axis could be important in the cross-talk between CD161 and LLT1 expressing immune cells.[17,23] This study was not designed to explore bidirectional interaction between CD161 expressed on gluten-reactive CD4+ T cells and LLT1 expressed on activated APCs. However, it is tempting to speculate that such interaction might be involved in fine tuning the interplay between gluten-reactive CD4+ T cells and gluten-specific and TG2-specific B cells in course of immune response to cereal gluten in CeD individuals.[30] Future studies are needed to decipher the role of CD161 on gluten-reactive CD4+ T cells.

Ethic statement

The study was approved by regional ethics committee (REK ID: 6544, project leader Knut E. A. Lundin).

Author contributions

LW design the study, performed the experiments, analysed and interpreted the data and wrote the manuscript; SW performed the experiments, interpreted the data and wrote the manuscript; LMS contributed to supervision and critical review of the manuscript. LW, LMS and SW approved the final version of the manuscript.

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Figure legends

Figure 1. CD161 is expressed on gluten-reactive CD4+ T cells. (A) Tetramer+ gluten-reactive CD4+ T cells from UCeD blood and gut were positive for CD161 (left panel). CD161 was expressed by higher proportion of tetramer+ gluten-reactive CD4+ T cells when compared to control tetramer- CD4+ T cells from the same tissue compartment (right panel). (B) The T-cell clones, that were generated from small intestinal specimens of treated CeD patients, expressed CD161. Gating strategy used to determine CD161 surface expression on T-cell clones was established based on fluorescence minus one control (FMO). (A and B) Numbers represent the percentage of gated cells.

Figure 2. Downregulation of surface CD161 upon ligation. Surface CD161 was downregulated upon ligation on two representative T-cell clones: TCC411.3.39 (upper panel) and TCC387.3 (lower panel). T-cell clones were stimulated with plate-bound anti-CD3 (1 μg/mL) and soluble anti-CD28 (5 μg/mL). Different anti-CD161 clones or matching isotype control were added either in soluble form (5 μg/mL) or immobilized on MACSiBead particles, as indicated. Surface CD161 expression was presented as the percentage of maximum CD161 expression. Data are representative of two independent experiments.

Figure 3. Ligation of CD161 does not increase cytokine production by TCR-stimulated T-cell clones. (A) T-cell clones were stimulated with plate-bound anti-CD3 (1 μg/mL) and soluble anti-CD28 (5 μg/mL). Different anti-CD161 clones or matching isotype control were added either in soluble form (5 μg/mL) or immobilized on MACSiBead particles, as indicated. Percentage of IFN-γ-secreting cells was measured by intracellular staining after 4 hrs and 6 hrs of stimulation. (B) T-cell clone TCC544.1.3.1 was stimulated with cognate gluten peptide (10 μM) presented by EBV-transformed lymphoblastoid B cells. Anti-CD161 (clone HP-3G10; 5 μg/mL) or appropriate isotype control (5 μg/mL) were added in a soluble version. IFN-γ production was measured as above. (A and B) Data are representative of two independent experiments.

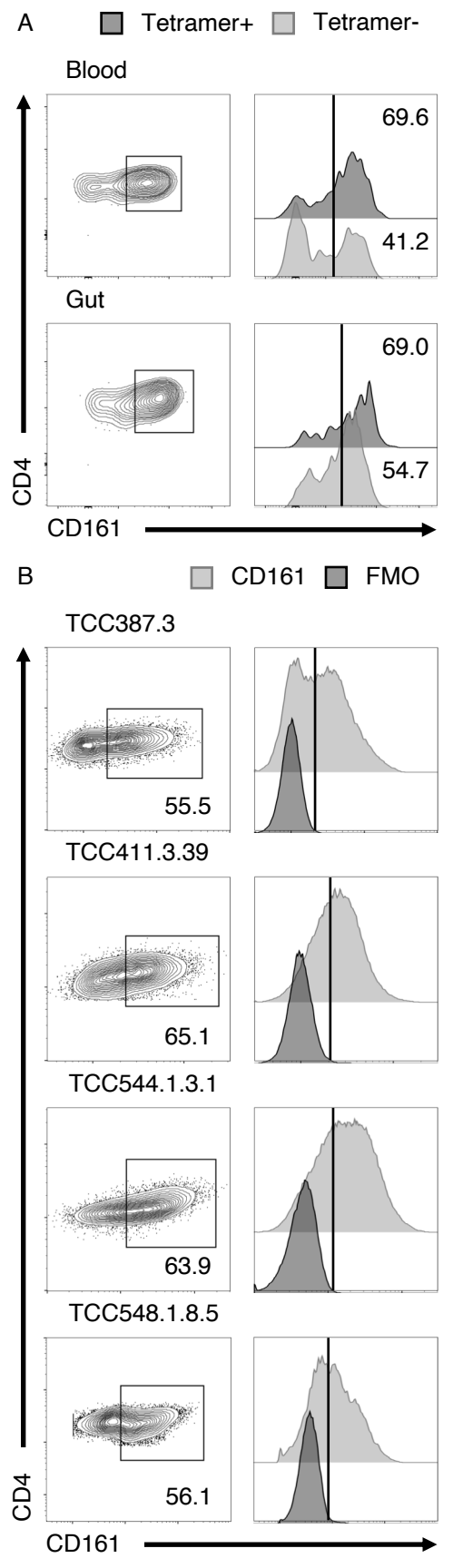
Figure 4. Ligation of CD161 does not influence proliferation of T-cell clones. (A) T-cell clones TCC544.1.3.1 (left) and TCC548.1.8.5 (right) were incubated for 72 hrs with plate-bound anti-CD3 and soluble anti-CD28 in concentrations as indicated. (B) T-cell clone TCC544.1.3.1 was stimulated for 72 hrs with cognate gluten peptide (in concentrations as indicated) presented by EBV-transformed lymphoblastoid B cells. (A and B) Anti-CD161 (clone HP-3G10; 5 μg/mL) or appropriate isotype control (5 μg/mL) were added in a soluble version.

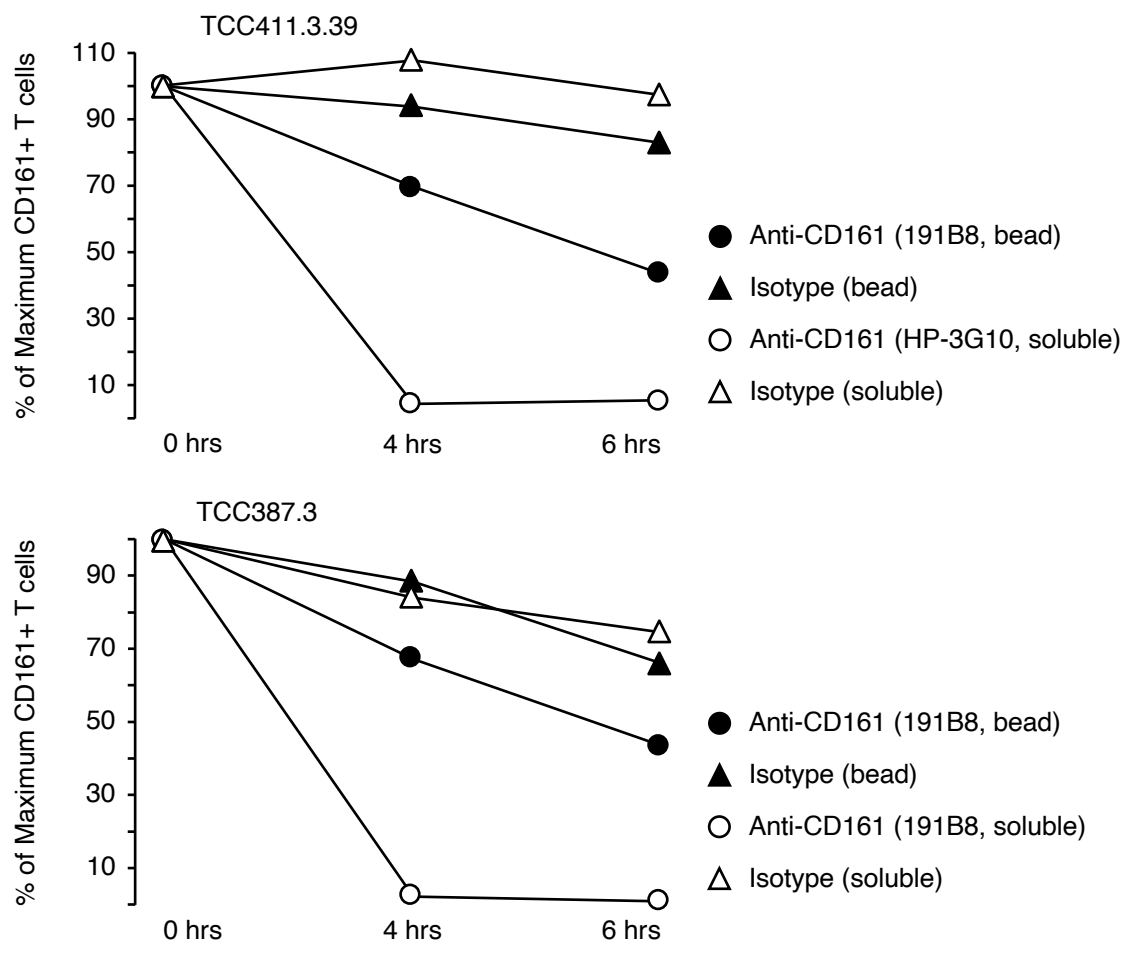
Assays were performed in triplicates and proliferation was measured by 3H-thymidine incorporation shown in counts per minute (cpm). Mean values ± SEM are shown.

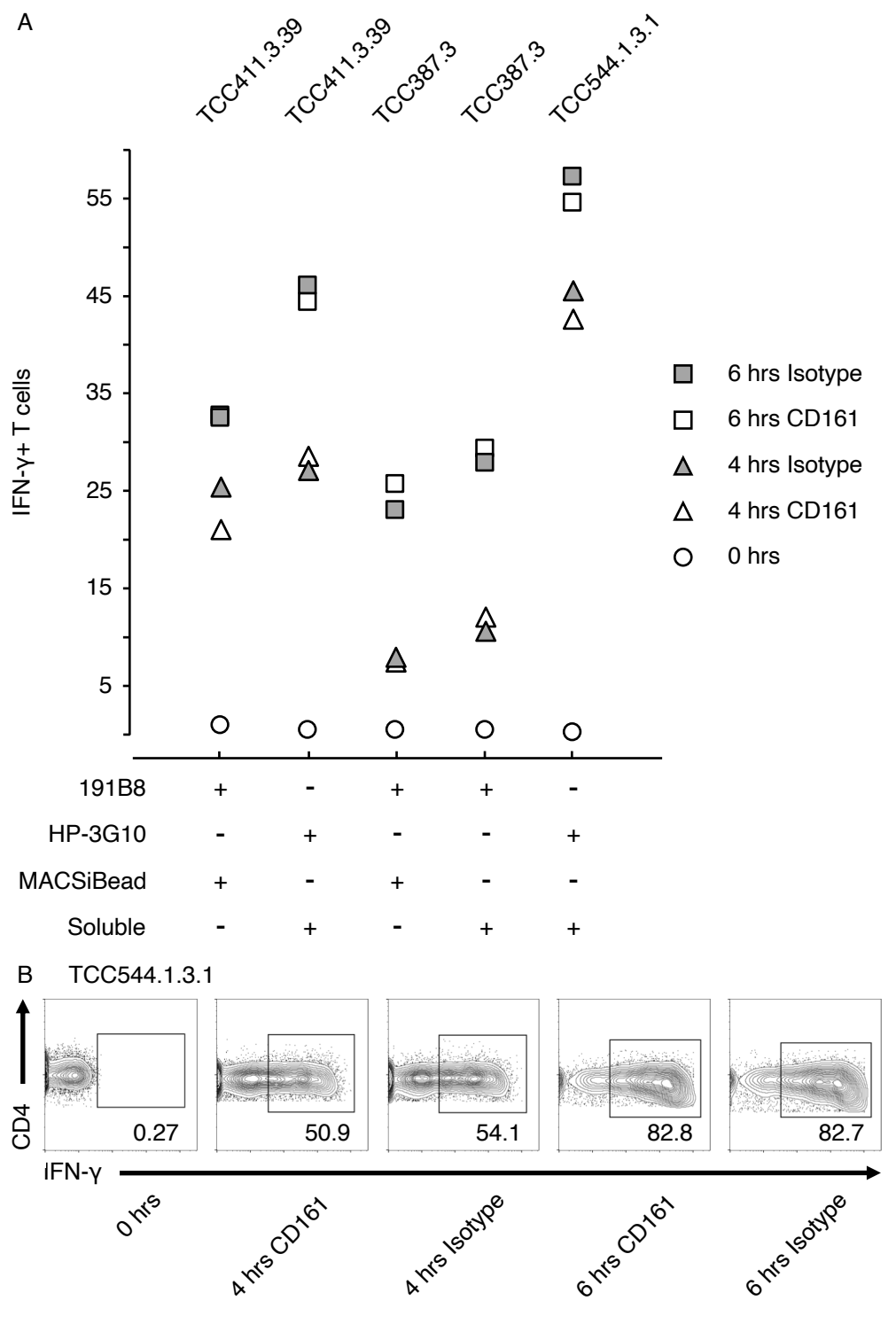
Supplementary Figure 1. Gating strategy. (A) Flow cytometric contour plots illustrate the gating strategy for gluten-reactive CD4+ T cells from blood and intestinal biopsies of one UCeD patient. Gating of cells from blood was done on lymphocytes → CD3+ T cells → CD4+ T cells → effector memory T cells (CD62L- CD45RA-). Gating of cells from gut was done on lymphocytes → CD3+ T cells → CD4+ T cells. (B) Gating of T-cell clones was done on lymphocytes → CD4+ T cells. Channel "dump" includes LIVE/DEAD cell viability marker, CD14, CD19, CD56 and CD11c. Abbreviations: FSC-A: Forward scatter areal; SSC-A: Side scatter areal; FSC-H: Forward scatter height; SSC-H: Side scatter height.

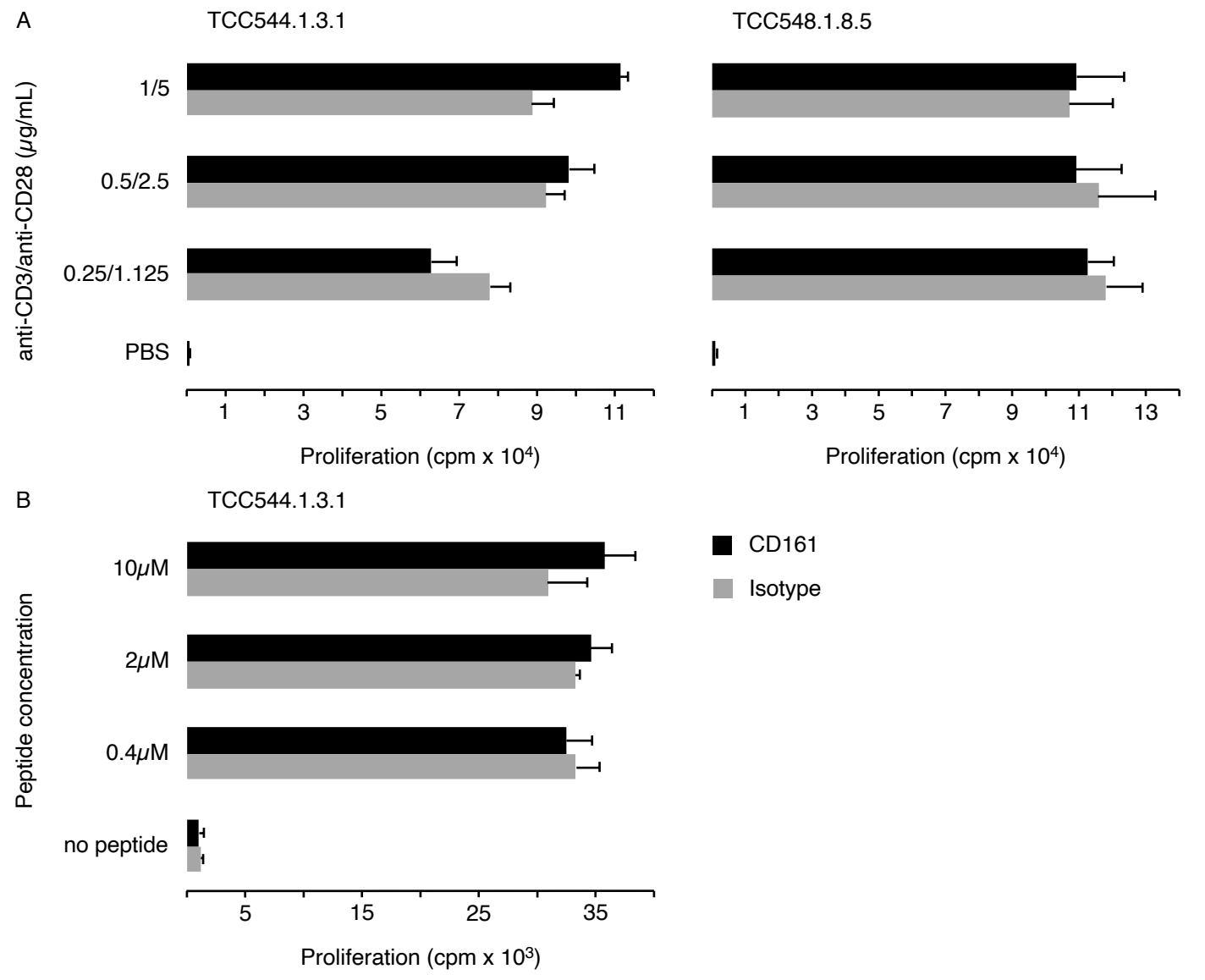
Supplementary Figure 2. Downregulation of surface CD161 upon ligation is dose-dependent. Surface CD161 was downregulated upon ligation on two representative T-cell clones: (A) TCC544.1.3.1 and (B) TCC548.1.8.5. T-cell clones were stimulated with plate-bound anti-CD3 (1 μg/mL) and soluble anti-CD28 (5 μg/mL). Soluble anti-CD161 (clone 191B8 or HP-3G10) were added at concentration as indicated in the legend. Matching isotype control were added in soluble form. Titration of isotype control caused negligible differences in surface CD161 downregulation (data not shown) and therefore the mean value was presented. (C) CD161 surface expression on fixed T-cell clones decreased upon pre-incubation with soluble anti-CD161 clone 191B8 or HP-3G10 used in saturating concentration of 5 μg/mL Two representative T-cell clones are shown. Surface CD161 expression was presented as the percentage of maximum CD161 expression.

Supplementary Figure 3. (A) Ligation of CD161 did not influence IL-21 production by T-cell clone TCC387.3. Cells were stimulated for 4 hrs and 6 hrs with plate-bound anti-CD3 (1 μg/mL), soluble anti-CD28 (5 μg/mL) and anti-CD161 (clone 191B8) or isotype control coated either on MACSiBead particles (upper panel) or added in a soluble version at saturating concentration of 5 μg/mL (lower panel). Numbers represent the percentage of gated cells. Data are representative of two independent experiments. (B) Gating strategy used for EBV-transformed lymphoblastoid B cell-line (left panel). Gating was done on single cells → lymphocytes → CD19+ B cells (left panel). EBV-transformed lymphoblastoid B cells were negative for LLT1 and CD161 (right panel). Gating strategy used to determine LLT1 and CD161 surface expression was established based on fluorescence minus one control (FMO). Abbreviations: FSC-H: Forward scatter height; FSC-A: Forward scatter areal; SSC-A: Side scatter areal.

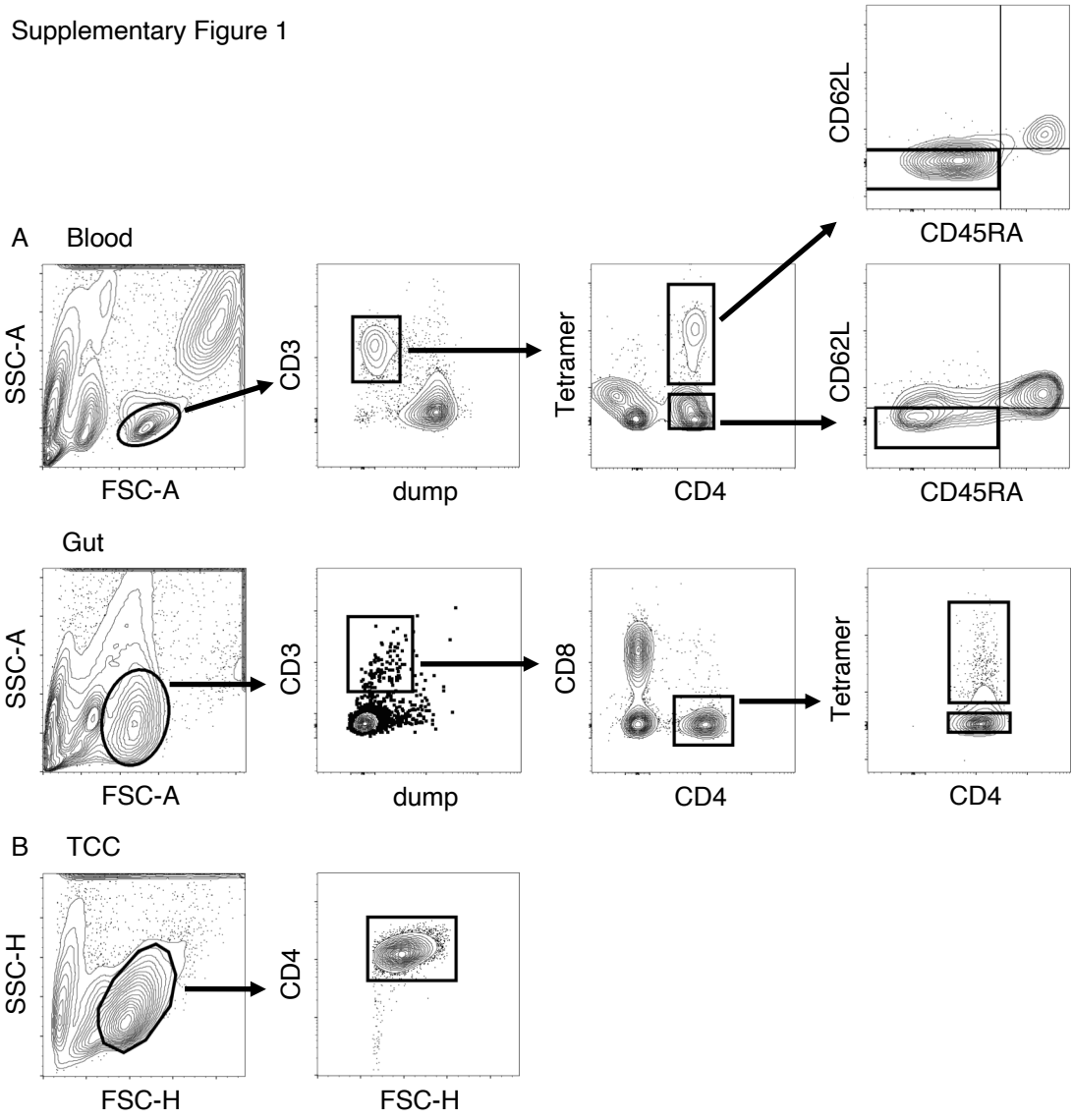

A
Tetramer+
Tetramer-
Blood
69.6
41.2
Gut
69.0
54.7
CD4
CD161
B
CD161
FMO
TCC387.3
55.5
TCC411.3.39
65.1
TCC544.1.3.1
63.9
TCC548.1.8.5
56.1
CD4
CD161


TCC411.3.39
% of Maximum CD161+ T cells
110
90
70
50
30
10
0 hrs
4 hrs
6 hrs
Anti-CD161 (191B8, bead)
Isotype (bead)
Anti-CD161 (HP-3G10, soluble)
Isotype (soluble)
TCC387.3
% of Maximum CD161+ T cells
90
70
50
30
10
0 hrs
4 hrs
6 hrs
Anti-CD161 (191B8, bead)
Isotype (bead)
Anti-CD161 (191B8, soluble)
Isotype (soluble)

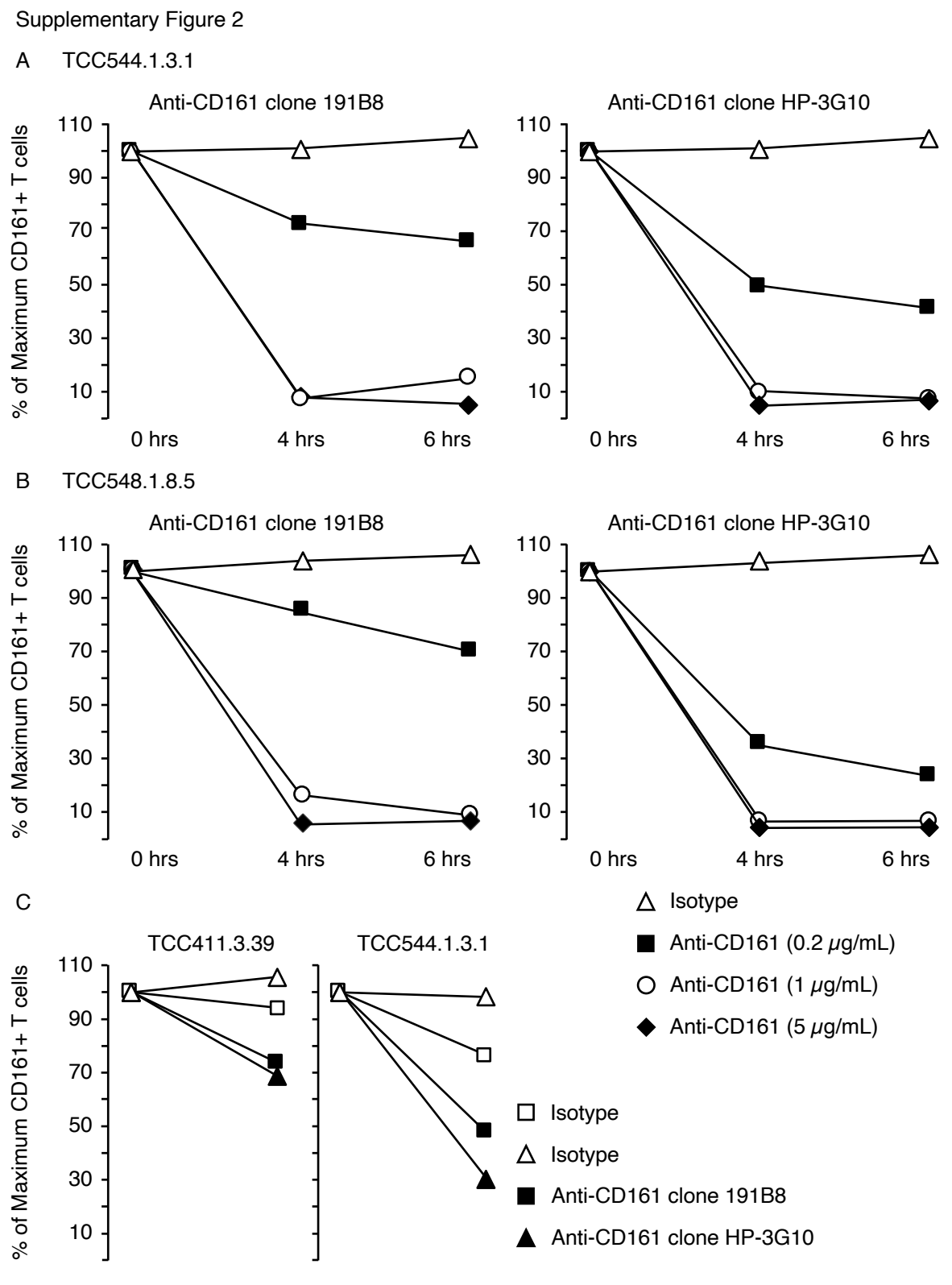

A
TCC411.3.39
TCC411.3.39
TCC387.3
TCC387.3
TCC544.1.3.1
IFN-γ+ T cells
55
45
35
25
15
5
6 hrs Isotype
6 hrs CD161
4 hrs Isotype
4 hrs CD161
0 hrs
191B8 + - + + -
HP-3G10 - + - - +
MACSiBead + - + - -
Soluble - + - + +
B
TCC544.1.3.1
CD4
IFN-γ
0.27
50.9
54.1
82.8
82.7
0 hrs
4 hrs CD161
4 hrs Isotype
6 hrs CD161
6 hrs Isotype


A
TCC544.1.3.1
TCC548.1.8.5
anti-CD3/anti-CD28 (μg/mL)
1/5
0.5/2.5
0.25/1.125
PBS
1
3
5
7
9
11
13
Proliferation (cpm x 10⁴)
B
TCC544.1.3.1
Peptide concentration
10μM
2μM
0.4μM
no peptide
CD161
Isotype
5
15
25
35
Proliferation (cpm x 10³)

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3