Received Date : 04-Jun-2012

Revised Date : 10-Aug-2012

Accepted Date : 20-Aug-2012

Article type : Original Article

Differential regulation and impact of fucosyltransferase VII and core 2 (beta) 1,6-N-acetyl-glycosaminyltransferase for generation of E- and P-selectin ligands in murine CD4$^+$ T cells

***Running title*:** Differential regulation of FucT-VII and C2GlcNAcT-I

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***Keywords*:** T cell activation, IL-2, P-selectin ligand, fucosyltransferase

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Abbreviations used: E-lig, E-selectin ligand; P-lig, P-selectin ligand; fucosyltransferase VII, FucT-VII; core 2 (beta) 1,6-glycosaminyltransferase I, C2GlcNAcT-I; CsA, cyclosporine A; CFSE: 5-(and 6-)carboxy-fluorescein diacetate succinimidyl ester

Summary

Ligands for E- and P-selectin (E- and P-lig) are induced on $CD4^+$ T cells upon differentiation into effector T cells. Glycosyltransferases, especially (alpha) 1,3 fucosyltransferase VII (FucT-VII) and core 2 (beta)1,6-N-acetyl-glycosaminyltransferase I (C2GlcNAcT-I), are critical for their synthesis. We here analysed the signals that control the expression of E- and P-lig and mRNA coding for FucT-VII and C2GlcNAcT-I. In line with previous reports, we found that P-lig expression correlates to the regulation of C2GlcNAcT-I, while E-lig expression can occur at low levels of C2GlcNAcT-I mRNA but requires high FucT-VII mRNA expression. Interestingly, the two enzymes are regulated by different signals. Activation-induced C2GlcNAcT-I upregulation under permissive (Th1) conditions was strongly reduced by cyclosporine A (CsA), suggesting the involvement of T cell receptor-dependent, calcineurin/NFAT-dependent signals in combination with IL-12-mediated signals in the regulation of C2GlcNAcT-I. In contrast, expression of FucT-VII mRNA was not significantly inhibited by CsA. IL-4 inhibited the expression of FucT-VII while IL-2 as well as IL-7 were found to support induction of FucT-VII and E-lig. E- and P-selectin and their ligands initially appeared to have rather overlapping functions. These findings however, unravel striking differences in the regulation of either E-lig and P-lig expression, dictated by the dominance of FucT-VII and C2GlcNAcT-I, respectively, and their dependency on signals from either promiscuous or homeostatic cytokines (FucT-VII) or a strong TCR signal in combination with inflammatory cytokines in case of C2GlcNAcT-I.

Introduction

P- and E-selectin ligands are involved in the recruitment of leukocytes into inflamed tissues. While myeloid cells express the ligands constitutively, lymphocytes show a restricted expression on subsets of memory/effector cells.[1,2] Selectin binding epitopes are generated after posttranslational modification of protein carriers such as P-selectin glycoprotein ligand (PSGL)-1 with sLex oligosaccharides.[3] A critical step in the synthesis of these epitopes is the addition of carbohydrate side chains by the activity of a variety of glucosaminyltransferases. Fucosyltransferase VII (FucT-VII) and

core 2 (beta)1,6-N-acetyl-glycosaminyltransferase I (C2GlcNAcT-I) are particularly important for the generation of selectin binding epitopes in lymphocytes since deletion of either one of these transferases results in the almost complete lack of E- and P-lig´s on T lymphocytes.[4,5] Whereas both enzymes, FucT-VII and C2GlcNAcT-I, are required for the generation of P-lig, E-lig binding is observed to some extent also in the absence of C2GlcNAcT-I.[5]

Initial *in vitro* studies showed that P-selectin ligands are induced in $CD4^+$ T cells concomitant to the Th1 differentiation program.[6] Studies using human T cells suggested that FucT-VII is induced by IL-12 and counterregulated by IL-4.[1] In contrast, in the murine system, Lim et al. as well as White et al. showed by using STAT4-deficient T cells, which have impaired intracellular IL-12 signaling, that IL-12 rather induces C2GlcNAcT-I but not FucT-VII expression.[7,8] T-bet was also shown to regulate selectin binding acting mostly via induction of C2GlcNAcT-I, which was reflected by a reduced IL-12-dependent induction of P-lig but unimpaired E-lig induction in T-$bet^{-/-}$ T cells.[9,10] Altogether, these data appeared to suggest that C2GlcNAcT-I is regulated by cytokine signals, most specifically IL-12, whereas FucT-VII is controlled by T cell activation. The impact of TCR signaling on FucT-VII induction was investigated by Barry et al, who showed that FucT-VII induction in a PMA responsive human T cells line was reduced by combined treatment with the p38 mitogen-activated protein kinases (MAPK) inhibitor SB203580 and the MEK1/2 inhibitor PD98059.[11] As PMA and TCR activate Ras, involvement of this pathway was suggested and transfection of Jurkat T cells with a constitutively active form of H-Ras led to E-lig binding and corresponding FucT-VII activity.[12]

However, TCR stimulation does not only activate the MEK and Ras-Raf pathway but also leads to calcineurin-dependent phosphorylation of NFAT proteins which activate a number of target genes after nuclear translocation. To investigate the role of this signaling pathway, we first analysed the effect of cyclosporin A (CsA), an inhibitor of the calcineurin-mediated NFAT activation, on the induction of selectin ligands in T cells. While P-lig and C2GlcNAcT-I induction was severely reduced by CsA treatment under Th1 conditions, FucT-VII induction was, surprisingly, only marginally affected. Moreover, analysis of the role of various cytokines highlighted striking differences in the regulation of the two enzymes resulting also in differential regulation of P-versus E-lig. Together with

the TCR signal, IL-12 is the major inducer of C2GlcNAcT-I under Th1 conditions, In contrast, FucT-VII was primarily induced during the resting phase by IL-2 or IL-7 and suppressed by IL-4 and to some extent IL-12. These findings demonstrate a so far not recognized role of common gamma chain cytokines in the regulation of FucT-VII in activated T cells and suggest a functional dichotomy among the ligands for E- and P-selectin in that FucT-VII-dominated synthesis of E-lig is predominantly driven by homeostatic cytokines while C2GlcNAcT-I-dependent P-lig synthesis requires a strong TCR signal combined with inflammatory cytokines, notably IL-12.

Materials and Methods

Mice

Balb/c mice were purchased from Charles River (Sulzfeld, Germany). DO11.10 mice were a kind by D.Y.Loh (Washington University School of Medicine, St Louis, MO) and T-bet$^{-/-}$ mice by A. Radbruch (Deutsches Rheumaforschungszentrum, Germany). Mice were bred under specific pathogen free conditions in the Bundesinstitut für Risikobewertung (Berlin, Germany). All animal experiments were performed in accordance with institutional, state and federal guidelines.

Antibodies, magnetic beads, culture media and cytokines

The following antibodies were produced in our laboratory or the Deutsche Rheumaforschungszentrum (Berlin, Germany): anti-CD3 (145-2C11), anti-CD28 (37.51), anti-IL-4 (11B11), anti-IL12 (C17.8), anti-IFNγ (AN18.17.26), anti-IL-2 (S4B6). Anti-CD4-FITC (L3T4), anti-CD62L-PE (MEL-14), anti-CD25-APC (PC61) and anti-CD90-PerCP (Ox-7) were purchased from BD Biosciences (Franklin Lakes, USA), anti-T-bet-APC (eBio4B10), anti-CD4-Alexafluor450 (L3T4), anti-CD62L-efluor450 (MEL-14) and isotype controls from eBioscience (Hatfield, UK), anti-CD43 (activated Glycoform)-PeCy7 (1B11) from BioLegend (San Diego, USA) and PE- conjugated F(ab´)$_2$ donkey anti-human IgG antibody from Jackson ImmunoResearch (West Grove, USA). All microbeads were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany).

Cell culture was set up in complete RPMI 1640 (Gibco Invitrogen, Carlsbad, USA) containing 10% FCS (Sigma-Aldrich, Taufkirchen, Germany), 5mM HEPES, penicillin and streptomycin, 1mM sodiumpyruvat (all from Biochrom AG, Berlin, Germany), and 10 µM 2-mercaptoethanol (Gibco Invitrogen, Carlsbad, USA). Recombinant murine IL-2, IL-7, IL-12, IFNγ, IL-4 and E-selectin-human IgG chimeric protein was purchased from R&D Systems (Wiesbaden, Germany). P-selectin-human IgG chimeric protein was kindly provided by M. Wild and D. Vestweber (Max Plank Institut für Vaskuläre Biologie, Münster, Germany).

Isolation and cell purification

For purification of naive $CD4^+$ T cells spleens, pLN and mLN were pooled and stained with anti-CD4-FITC mAb and anti-FITC multisort magnetic activated cell sorting (MACS) beads. Cells were sorted using an AutoMACS (Miltenyi Biotech) reaching a purity of at least 95%. After release of multisort beads according to the manufacturer instruction $CD62L^+$ T cells were isolated using anti-CD62L microbeads. In some experiments $CD25^+$ T cells were depleted using anti-CD25-APC and anti-APC beads before CD4 enrichment. Antigen presenting cells were prepared by depletion of $CD90^+$ cells from CD4-depleted cell fraction and irradiated (30 gray) before culture.

Cell culture

Naive OVA TCR^{tg} T cells were cultured with APCs at a ratio of 1:4 in the presence of 0,5µM $OVA_{323-339}$ peptide (Biochemistry Department, Charité Universitätsmedizin, Berlin, Germany). For polyclonal activation, sorted naïve T cells were cultured on plates coated with anti-CD3 and anti-CD28 mAbs at 1 x 10^6 cells/ml. For Th1 polarizing conditions cultures were supplemented with recombinant murine IL-12 at 5 ng/ml, IFNγ at 20 ng/ml and neutralizing anti-IL-4 mAb at 5 µg/ml. For Th2 polarizing conditions recombinant murine IL-4 at 30 ng/ml, neutralizing anti-IL-12 mAb at 5 µg/ml and neutralizing anti-IFNγ mAb at 5 µg/ml were added to the culture. For Th0 conditions neutralizing anti-IL-12 mAb, anti-IFNγ and anti-IL-4 mAb was added to the culture. IL-2 or IL-7 were added at 10 ng/ml as indicated. For cyclosporine A (CsA) treatment T cells were incubated for

20 min at 37°, 5% CO_2 with CsA (Sigma Aldrich) before cytokines, antibodies and stimulus were added.

5-(and6-)carboxy-fluorescein diacetate succinimidyl ester (CFSE) labeling

Naïve $CD4^+$ T cells were washed with PBS before labeling and resuspended at $1x10^7$ cells/ml in PBS containing 5µM CFSE (Molecular Probes). After incubation for 2.5 min at room temperature the reaction was stopped by adding RPMI 1640 with 10% FCS.

Cytometric analysis and FACS sorting

For cytometric analysis cells were stained with the respective antibodies for 20 min at 4 °C in the dark. P- and E-lig´s were detected with P- and E-selectin-human IgG chimeric proteins and PE-conjugated anti-human IgG Ab $F(ab')_2$ as a secondary reagent. Staining was performed in Ca^{2+} containing HBSS buffer supplemented with 10 mM Hepes as previously described.[13] A FACSCalibur, LSR II or LSRFortessa (all BD Biosciences, Franklin Lakes, NJ) and the CellQuest Pro (BD Biosciences) or FlowJo (Tree Star, Ashland, USA) software were used for FACS analysis.

For sorting of P-lig^+ and P-lig^- T cell populations from Th1 and Th2 cultures, naive T cells were stained with CFSE and cultured under Th1 or Th2 conditions. For sorting, cultured cells were stained for CD4 and P-lig. $CD4^+$ T cells were sorted according to P-lig expression into P-lig^+ and P-lig^- cell fraction. Dead cells were excluded according to propidium iodide or 4,6-diamidino-2-phenylindol staining (Sigma, St. Louis, MO). To ensure similar proliferation status of the sorted populations P-lig^+ and P-lig^- T cells from similar generations were sorted according to CFSE staining. Sorting was performed on a FACSAria or a FACSDiva (all BD Biosciences).

Quantitative PCR

Total RNA was isolated with RNeasy Mini Kit and QiaShredder (Qiagen) and DNA removed with RNase-Free DNase Set (Qiagen). RNA was reverse transcribed by Superscript II Reverse Transcriptase (Invitrogen) using oligo(dT) and random hexamer primer (Qiagen). Quantitative PCR

of FucT-VII or C2GlcNAcT-I mRNA was performed either as described[13] using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, USA) or by using the platinum SYBR Green qPCR Super-Mix-UDG (Invitrogen) and the same primer pairs and the Mx3000P or Mx3005P qPCR system (Agilent Technologies, Santa Clara, USA).

Statistics

The PRISM (GraphPad, San Diego, USA) software was used for statistical analysis.

Results

Impact of TCR-mediated signals on the regulation of P-lig and glycosyltransferases

As known, activation of naïve $CD4^+$ T cells under permissive (Th1) conditions leads to a strong induction of P-lig. However, surface expression of the C2GlcNAcT-I-dependent epitope 1B11 and the functional P-lig epitope as well as FucT-VII and C2GlcNAcT-I mRNA are rather slowly upregulated and reach high levels only at or from day 3 on (Figure 1A-C).

This raises the question whether T cell signals induce FucT-VII and C2GlcNAcT-I indeed directly or whether the regulation rather occurs indirectly, e.g. via cytokines or cytokine receptors. First, we exploited the heterogenous expression of the transgenic TCR in DO11.10 mice to analyze whether P-lig induction after activation of naïve $CD4^+$ T cells depends on a cell-intrinsic TCR signal or on changes in the milieu. As shown in Fig. 1D, P-lig induction was observed only on T cells expressing the transgenic OVA-specific TCR ($KJ1.26.1^+$). Secondly, we used cyclosporine A (CsA) which inhibits the calcineurin-dependent dephosphorylation and translocation of NFAT into the nucleus to block TCR-mediated activation. P-lig induction was dose-dependently inhibited by CsA (Figure 1E) confirming that TCR-dependent signals are indeed required for the induction of P-lig on T-cells as shown previously.[13] When T cells were activated under non-permissive conditions, i.e. Th2, we observed a low, but reproducible induction of P-lig on T cells treated with high concentrations of CsA (Figure 1F).

Inhibition of the calcineurin pathway suppressed P-lig induction beyond its inhibition of proliferation as seen when the impact of CsA on P-lig induction was analyzed in individual generations detected by loss of CFSE (Figure 2A). P-lig expression was also reduced within single generations of T cells activated under Th1 polarizing conditions suggesting that CsA treatment affects P-lig induction independently of its anti-proliferative action (Figure 2B).

Thus, TCR/calcineurin-mediated, cell-intrinsic signals are essential for the induction of P-lig. To determine whether the TCR signal regulates FucT-VII, C2GlcNAcT-I or both we sorted cells of generations 2 and 3 on day 3 after activation and determined mRNA levels or the glycosyltransferases. Surprisingly, CsA treatment only slightly reduced FucT-VII mRNA expression in T cells cultured either under Th1 or Th2 conditions (Figure 2C). In contrast, CsA treatment affected C2GlcNAcT-I in the same way as P-lig expression: it suppressed C2GlcNAcT-I expression in Th1-polarized cells but allowed slightly increased levels in proliferated Th2-polarized cells (Figure 2D). This shows that C2GlcNAcT-I expression dictates the expression of P-lig under these conditions. Also the paradoxical effect of CsA on P-lig expression induced under Th2 conditons was found to correlate to C2GlcNAcT-I, but not FucTVII mRNA expression.

The contrasting effect of CsA treatment on C2GlcNAcT-I under Th1 and Th2 conditions suggests that TCR signalling differentially regulates C2GlcNAcT-I expression depending on signals from polarizing cytokines.

C2GlcNAcT-I mRNA expression dictates the expression of P-lig in Th cells

As apparent from Figure 1 and 2, only a fraction of T cells expresses P-lig in Th1 cultures and, an even smaller fraction in Th2 cultures. To analyze whether P-lig$^+$ and P-lig$^-$ fractions differ in their expression of FucT-VII and/or C2GlcNAcT-I mRNA, we sorted P-lig$^+$ and P-lig$^-$ T cells from Th1 and Th2 cultures. Th1 cells were sorted from day 3-cultures, a time point with intermediate levels of P-lig$^+$ cells (see following Figure 4) allowing purification of P-lig$^+$ and P-lig$^-$ T cells fraction. Under Th2 conditions, P-lig expression was low but stable from day 3 to day 5. As total numbers of P-lig$^+$ in Th2 cultures were higher at day 5 due to cellular expansion this time point alleviating acquisition of

the rare P-lig$^+$ Th2 cells, was chosen to sort Th2 cultures into P-lig$^+$ and P-lig$^-$ fraction. To exclude any bias due to differences in proliferation, P-lig$^+$ and P-lig$^-$ fractions were again sorted from similar generations, i.e. generation 2 and 3, according to CFSE loss (Figure 3A). FucT-VII and C2GlcNAcT-I mRNA was upregulated in both P-lig$^+$ and P-lig$^-$ fractions compared to naive T cells (Figure 3B). However, whereas FucT-VII mRNA levels did only slightly differ between Th1-P-lig$^+$ and P-lig$^-$ cells and not among these fractions of Th2 cells, C2GlcNAcT-I mRNA was much stronger expressed in P-lig$^+$ Th1 and Th2 cells. This again suggests that C2GlcNAcT-I is the key regulator of P-lig expression under these conditions. Furthermore, it also suggests that both enzymes are differentially regulated.

Differential regulation of FucT-VII and C2GlcNAcT-I by cytokine signals

To determine the impact of cytokines on C2GlcNAcT-I and FucT-VII regulation we activated naive CD4$^+$ cells either under Th1, Th2 or Th0 conditions for 3 days by anti-CD3/anti-CD28 mAb followed by a 2-day resting phase. IL-2 was added to all cultures. In addition to P-lig we also determined E-lig expression in order to correlate expression of the glycosyltransferases C2GlcNAcT-I and FucT-VII to P-lig and E-lig expression.

As known, P-lig induction was most efficient under Th1 conditions and evident already 3 days after activation. This was associated with high C2GlcNAcT-I expression (Figure 4A and C). In contrast, high E-lig expression was found on day 5 after activation in Th1 and Th0 cultures which was accompanied by high FucT-VII expression (Figure 4B and D). In contrast to C2GlcNAcT-I, which peaked on day 3, FucT-VII increased up to day 5 in Th1 and Th0 cultures after removal from the TCR stimulus and further culturing in the presence of IL-2 (Figure 4D). These data confirm that C2GlcNAcT-I determines expression of P-lig whereas E-lig expression is controlled by FucT-VII and can occur in the absence of higher levels of C2GlcNAcT-I induction.

To analyse the impact of individual cytokines on FucT-VII and C2GlcNAcT-I mRNA induction we cultured naive T cells with IL-2 and additional distinct combinations of IL-12, IFNγ and IL-4 and/or blocking antibodies. IFNγ or IL-4 alone or their blockade during IL-12-dependent induction did not significantly affect C2GlcNAcT-I induction confirming that IL-12 is the major inducer of

C2GlcNAcT-I (Figure 5A). Strikingly, FucT-VII mRNA expression was most strongly induced under Th0 conditions, and significantly reduced in the presence of IL-4 (Figure 5B). Addition of IL-12, but not the combination of IL-12 and IFNγ, i.e. Th1 conditions, also resulted in significantly lower FucT-VII expression than under Th0 conditions (Figure 5B).

T-bet is induced by IFNγ and IL-12 signaling.[14] Since previous studies suggested that T-bet is involved in C2GlcNAcT-I induction [9,10] we analyzed the induction of P-lig, FucT-VII and C2GlcNAcT-I mRNA in T-bet$^{-/-}$ T cells 3 days after stimulation under Th1 conditions. However, we found no significant difference in P-lig, C2GlcNAcT-I and FucT-VII expression in T-bet-deficient cells compared to wildtype controls (Figure 5C) at this early time point suggesting that T-bet induced by T cell activation or IFNγ is not involved in the primary steps of the IL-12-dependent-induction of C2GlcNAcT-I.

Blockade of IL-2 inhibits FucT-VII expression

To confirm the hitherto not reported role of IL-2 for the regulation of FucT-VII, we stimulated naive T cells under Th0 or Th1 conditions for 3 days and added from day 3 on either exogenous IL-2 or a neutralizing anti-IL-2 mAb. Blockade of IL-2 reduced E-lig as well as FucT-VII upregulation in T cells cultured under Th0 conditions (Figure 6A). IL-7, which also signals via the common gamma chain of the IL-2 receptor, could restore FucT-VII mRNA expression (Figure 5A). Expression of P-lig and C2GlcNAcT-I, which is low under these conditions, was unaffected by IL-2 blockade (Figure 5A). Under Th1 conditions, IL-2 blockade also resulted in reduced expression of E-lig and in tendency also of FucT-VII (Figure 6B). IL-7 could at least partially rescue the effect on E-lig expression, while IL-12 and IFNγ present under Th1 conditions were obviously unable to do so (Figure 6B). P-lig as well as C2GlcNAcT-I mRNA expression were unaffected by IL-2 blockade.

These data demonstrate that, in addition to TCR signal, IL-12 is a key inducer of C2GlcNAcT-I and P-lig, but not FucT-VII expression, while IL-2 and the homeostatic cytokine IL-7 are important regulators of FucT-VII and E-lig.

Discussion

By analysing glycosyltransferase expression in P-lig$^+$ and P-lig$^-$ activated T cells we show that C2GlcNAcT-I rather than FucT-VII expression determines P-lig expression. For instance, C2GlcNAcT-I expression was much higher in P-lig$^+$ Th1 versus P-lig$^-$ Th1 cells. Moreover, lack or abrogation of C2GlcNAcT-I induction, as for instance under Th0 conditions or after CsA treatment, correlated to low P-lig induction. However, residual expression or low induction of FucT-VII still seems to be required for P-lig synthesis since Th1 cells from FucT-VII$^{-/-}$ mice lack P-lig expression and show impaired recruitment into acute inflamed DTH-skin sites.[4,15] In contrast, E-lig expression was found in T cells in the absence of C2GlcNAcT-I induction such as under Th0 conditions which, however, required high levels of FucT-VII mRNA expression. For E-lig synthesis, even residual C2GlcNAcT-I expression might be dispensable as granulocytes and Th1 cells from C2GlcNAcT-I knockout mice retain some binding to E-selectin although binding to P-selectin is abolished.[5]

Apart from the distinct contribution of FucT-VII and C2GlcNAcT-I to the generation of P-lig and E-lig in CD4$^+$ T cells, the data of our study also show that the two glycosyltransferases are differentially regulated by cytokines and the TCR signal.

First, in agreement with other reports, we found that C2GlcNAcT-I induction is regulated primarily by polarizing cytokines, particularly IL-12, while T cell activation in the presence of IL-2 (Th0 conditions) was not sufficient to induce C2GlcNAcT-I expression.[7,8] Moreover, blockade of IL-2 in expanding, activated T cells did not affect C2GlcNAcT-I expression. IL-4 had no direct suppressive effect on C2GlcNAcT-I expression and in contrast to other studies, we found no major inducing effect of IFNγ and T-bet on the expression of P-lig and C2GlcNAcT-I.[9,10] However, in the study by Underhill et al. the differences between WT and T-bet$^{-/-}$ T cells were most prominent after prolonged culture.[9] As we only analysed early time points our data do not preclude that T-bet acts at later time points for instance by stabilising the expression of C2GlcNAcT-I.

In contrast, IL-4 suppressed FucT-VII induction. as shown before by Wagers et al..[1] Yet, in contrast to this study which found induction of FucT-VII by IL-12 we observed that IL-12 in the absence of IFNγ

suppressed FucT-VII expression, though less drastic than IL-4.[1] However, addition of IFNγ resembling Th1 culture conditions, seems to prevent the inhibitory effect of IL-12 resulting in similar levels of FucT-VII expression as under Th0 conditions. Considering that some of the cultures in the Wagers study were done with total $CD4^+$ T cells and without blockade of IFNγ which rather reflects Th1 conditions both studies concur that Th1 conditions support high FucT-VII induction.

IL-2, however, promoted high FucT-VII expression in activated T cells and blockade of IL-2 abrogated induction of E-lig and FucT-VII. In particular FucT-VII mRNA expression could be rescued by IL-7, another common gamma chain cytokine [16] which suggests that promiscous and homeostatic cytokines play a hitherto unknown role in the induction of FucT-VII in activated T cells. The rescue of E-lig expression by IL-7 was not as effective as the restoration of FucT-VII mRNA expression suggesting that IL-2 and IL-7 might affect other parts of the E-lig synthesis pathway with differing efficacy.

Differential effects on FucT-VII and C2GlcNAcT-I expression were also observed after blockade of calcineurin-dependent TCR signals. TCR signaling, in particular, p38 and Ras-Raf dependent pathways were shown before to control FucT-VII induction.[11,12] We found that blocking calcineurin-dependent pathways of T cell activation by CsA rather inhibited C2GlcNAcT-I but not FucT-VII mRNA induction. This is surprising, since, CsA by inhibiting calcineurin-dependent signals also inhibits the production of IL-2 which, we found here, is an inducer of FucT-VII.[17] However, the inducing effect of IL-2 on FucT-VII was most obvious between day 3 and day 5 of culture, i.e. after removal of the cells from the TCR stimulus, suggesting that IL-2 and IL-7 which can substitute IL-2 acts at later time points and Ras-Raf dependent pathways, possibly less affected by the CsA treatment, might control the early TCR-dependent FucT-VII induction. Alternatively, minute amounts of IL-2 produced even in the presence of CsA might be sufficient to induce FucT-VII.

Moreover, in a previous study we showed that FucT-VII induction in naïve T cells requires cell cycling which is induced by the TCR stimulus.[13] This, and the fact that naive T cells express IL-7

receptors but do not express FucT-VII nor E-lig suggest another level of regulation such as epigenetic modification of the *fucosyltransferase 7* gene locus.

In contrast to FucT-VII, CsA treatment inhibited the induction of C2GlcNAcT-I under Th1 conditions. Although we cannot completely exclude that the effect of CsA is due to impaired availability of IL-2 this possibility appears unlikely since IL-2 did not support C2GlcNAcT-I induction under Th0 conditions and IL-2 blockade had no major effect on C2GlcNAcT-I expression in activated T cells. It rather suggests direct involvement of NFAT-dependent signals. However, NFAT phosphorylation occurs early after TCR triggering but induction of C2GlcNAcT-I and hence the inhibitory effect of CsA is just observed after 48 to 72 hours after activation. Thus, the effect of CsA on C2GlcNAcT-I induction might be indirect by NFAT-dependent regulation of other pathways, in particular IL-12-dependent signals. However, IL-12Rbeta2 expression appeared to be unaffected by CsA treatment (data not shown) and T-bet was shown to be dispensable for this early C2GlcNAcT-I expression suggesting that NFAT proteins might even directly cooperate with STAT4-dependent signals to induce C2GlcNAcT-I expression. The differential effect of CsA on FucT-VII and C2GlcNAcT-I could therefore indeed reflect dichotomy in the requirement of distinct downstream TCR signaling pathways, i.e. NFAT and Ras-Raf dependent pathways, for C2GlcNAcT-I and FucT-VII induction.

Surprisingly, CsA treatment resulted in slightly increased expression of P-lig in progressed T cells cultured under Th2 polarizing conditions, i.e. in the presence of IL-4. This can not be explained by changes in IL-4 signaling due to CsA treatment as IL-4 did not actively suppress C2GlcNAcT-I mRNA expression (Figure 4). It rather suggests that restraining NFAT-dependent TCR signals under Th2 conditions turns IL-4 into an inducer of C2GlcNAcT-I. Such IL-4 dependent induction mechanisms are likely to exist as *in vivo* the majority of IL-4-producing $CD4^+$ T cells coexpress P-lig.[18]

Altogether our data show striking difference in the requirement of FucT-VII and C2GlcNAcT-I for P-lig and E-lig expression in $CD4^+$ T cells. Moreover, we show that both enzymes are differentially

regulated. While IL-12 and a strong TCR signal upregulate C2GlcNAcT-I, FucT-VII is induced independent from calcineurin-dependent NFAT signals by homeostatic cytokines such as IL-2 and IL-7. The promoting action of IL-2 and IL-7, both binding to the common gamma chain of IL-2 receptor, suggest that STAT5 signalling is involved in transcriptional activation of *fucosyltransferase 7*.

Conflict of interest

The authors do not have any conflicts of interests.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Transregio 52 TP B4). We thank H. Schliemann, H. Hecker, and T. Geeske for providing us with mAbs.

References

1. Wagers AJ, Waters CM, Stoolman LM, Kansas GS. Interleukin 12 and interleukin 4 control T cell adhesion to endothelial selectins through opposite effects on alpha1, 3-fucosyltransferase VII gene expression. J Exp Med 1998; 188(12):2225-31.

2. Lim YC, Henault L, Wagers AJ, Kansas GS, Luscinskas FW, Lichtman AH. Expression of functional selectin ligands on Th cells is differentially regulated by IL-12 and IL-4. J Immunol 1999; 162(6):3193-201.

3. Lowe JB. Glycan-dependent leukocyte adhesion and recruitment in inflammation. Curr Opin Cell Biol 2003; 15(5):531-8.

4. Smithson G, Rogers CE, Smith PL*, et al.* Fuc-TVII is required for T helper 1 and T cytotoxic 1 lymphocyte selectin ligand expression and recruitment in inflammation, and together with Fuc-TIV regulates naive T cell trafficking to lymph nodes. J Exp Med 2001; 194(5):601-14.

5. Snapp KR, Heitzig CE, Ellies LG, Marth JD, Kansas GS. Differential requirements for the O-linked branching enzyme core 2 beta1-6-N-glucosaminyltransferase in biosynthesis of ligands for E- selectin and P-selectin. Blood 2001; 97(12):3806-11.

6. Austrup F, Vestweber D, Borges E*, et al.* P-and E-selectin mediate recruitment of T helper 1 but not T helper 2 cells into inflamed tissues. Nature 1997; 385(6611):81-3.

7. Lim Y, Xie H, Come C, Alexander S, Grusby M, Lichtman A, Luscinskas F. IL-12, STAT4-dependent up-regulation of CD4(+) T cell core 2 beta-1,6-n-acetylglucosaminyltransferase, an enzyme essential for biosynthesis of P-selectin ligands. J Immunol 2001; 167:4476-84.

8. White SJ, Underhill GH, Kaplan MH, Kansas GS. Cutting edge: differential requirements for Stat4 in expression of glycosyltransferases responsible for selectin ligand formation in Th1 cells. J Immunol 2001; 167(2):628-31.

9. Underhill GH, Zisoulis DG, Kolli KP, Ellies LG, Marth JD, Kansas GS. A crucial role for T-bet in selectin ligand expression in T helper 1 (Th1) cells. Blood 2005; 106(12):3867-73.

10. Lord GM, Rao RM, Choe H, Sullivan BM, Lichtman AH, Luscinskas FW, Glimcher LH. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. Blood 2005; 106(10):3432-9.

11. Barry SM, Zisoulis DG, Neal JW, Clipstone NA, Kansas GS. Induction of FucT-VII by the Ras/MAP kinase cascade in Jurkat T cells. Blood 2003; 102(5):1771-8.

12. Zisoulis DG, Kansas GS. H-Ras and phosphoinositide 3-kinase cooperate to induce alpha(1,3)-fucosyltransferase VII expression in Jurkat T cells. J Biol Chem 2004; 279(38):39495-504.

13. Syrbe U, Jennrich S, Schottelius A, Richter A, Radbruch A, Hamann A. Differential regulation of P-selectin ligand expression in naïve versus memory T cells: evidence for epigenetic regulation of involved glycosyltransferase genes. Blood 2004; 104:3243-8.

14. Schulz EG, Mariani L, Radbruch A, Hofer T. Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. Immunity 2009; 30(5):673-83.

15. Doebis C, Siegmund K, Loddenkemper C, Lowe JB, Issekutz AC, Hamann A, Huehn J, Syrbe U. Cellular players and role of selectin ligands in leukocyte recruitment in a T-cell-initiated delayed-type hypersensitivity reaction. Am J Pathol 2008; 173(4):1067-76.

16. Liao W, Lin JX, Leonard WJ. IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. Curr Opin Immunol 2011; 23(5):598-604.

17. Podtschaske M, Benary U, Zwinger S, Hofer T, Radbruch A, Baumgrass R. Digital NFATc2 activation per cell transforms graded T cell receptor activation into an all-or-none IL-2 expression. PLoS ONE 2007; 2(9):e935.

18. Kretschmer U, Bonhagen K, Debes GF*, et al.* Expression of selectin ligands on murine effector and IL-10-producing CD4(+) T cells from non-infected and infected tissues. Eur J Immunol 2004; 34(11):3070-81.

Figure Legends:

Figure 1: TCR signaling is required for induction of P-lig

Naive T cells from Balb/c mice were activated by anti-CD3/ anti-CD28 stimulation under Th1 (IL-2 + IL-12 + IFNγ + anti-IL-4) conditions. 1B11 and P-lig expression (staining example A, summary B) and FucT-VII and C2-GlcNAcT-I mRNA expression (C) were determined at indicated time points of culture. CFSE labeled naive $CD4^+$ T cells from DO11.10 mice were activated in the presence of $OVA_{323-339}$ peptide, APC and Th1 polarizing cytokines (IL-12 + IFNγ + anti-IL-4) or Th2 polarizing conditions (anti-IFNγ + anti-IL-12 + IL-4). 3 days after activation $CD4^+$ cells were stained for expression of the transgenic OVA-specific TCR using the clonotype specific antibody KJ1.26.1 (D).

Proliferation determined by loss of CFSE and P-lig induction was analyzed for transgenic and non-transgenic T cells. CsA was added to polyclonally activated Th1 (IL-12 + IFNγ + anti-IL-4) and Th2 cultures (anti-IFNγ + anti-IL-12 + IL-4) at different concentrations (E and F). P-lig expression was determined on day 3 after activation ($n \geq 4$; mean ± SD. * $p<0.05$, **$p<0.01$, ***$p<0.001$; unpaired t-test)

Figure 2: Differential effect of CsA on P-lig induction under Th1 and Th2 polarizing conditions

CFSE labeled naive $CD4^+$ T cells from Balb/c mice were activated by anti-CD3/ anti-CD28 stimulation under Th1 (IL-12 + IFNγ + anti-IL-4) or Th2 polarizing (anti-IL-12 + anti-IFNγ + IL-4) conditions and different concentrations of CsA. In A) examples of P-lig and CFSE stainings, performed on day 3 after activation and the gating of individual generations are shown for Th1 (left panel) and Th2 cultures (right panel). In B) the summary of P-lig expression analyzed in generations 1-3 of Th1-polarized and Th2 polarized cells is shown (Th1: $n \geq 6$; Th2: $n = 4$). In C) FucT-VII and C2GlcNAcT-I mRNA expression for FACS-sorted cells of generation 3 ($n \geq 4$) (mean ± SD. * $p<0.05$, **$p<0.01$, ***$p<0.001$, n.s. not significant; unpaired t-test)

Figure 3: Differential expression of C2GlcNAcT-I mRNA in P-lig$^+$ and P-lig$^-$ cell fraction from Th1 and Th2 cultures

CFSE-labelled, naive $CD4^+$ T cells were activated by anti-CD3/anti-CD28 stimulation under Th1 (IL-2 + IL-12 + IFNγ + anti-IL-4) or Th2 (IL-2 + anti-IL-12 + anti-IFNγ + IL-4) conditions. On day 3 (Th1) or day 5 (Th2) after stimulation cells of generations 2 and 3 were sorted into P-lig$^+$ and P-lig$^-$ fractions (A, one representative sort of Th1 and Th2 cells is shown, numbers on the right side of the plot indicate percentage of P-lig$^+$ and P-lig$^-$ cells). mRNA expression of FucT-VII and C2GlcNAcT-I normalized to HPRT mRNA expression of these T cell fractions and naive T cells is given (B). (naive T cells, n=15; Th1 n=6; Th2, n=7; *$p<0.05$ and **$p<0.01$ vs. naive T cells, Mann Whitney U test, ###$p<0.001$, paired t test).

Figure 4: FucT-VII and E-lig expression is promoted under Th0 conditions in the absence of TCR stimulation

Naive $CD4^+$ T cells were activated by anti-CD3/anti-CD28 stimulation under Th1 (IL-2 + IL-12 + IFNγ + anti-IL-4), Th2 (IL-2 + anti-IL-12 + anti-IFNγ + IL-4) or Th0 (IL-2 + anti-IL-12 + anti-IFNγ + anti-IL-4) polarizing conditions. P-lig (A) and E-lig (B) expression as well as C2GlcNAcT-I (C) and FucT-VII mRNA (D) expression normalized to HPRT mRNA expression was determined on day 3 and day 5 after activation ($n \geq 3$; mean ± SD. ***$p<0.001$ vs. Th1 same day, **$p<0.01$ vs. Th1 same day; * $p<0.05$ vs. Th1 same day; unpaired t-test).

Figure 5: C2GlcNAcT-I is induced by IL-12, while FucT-VII is induced in the presence of IL-2 and suppressed by IL-4 and IL-12

Naive T cells were activated by anti-CD3/anti-CD28 stimulation in the presence of IL-2 and the indicated combinations of cytokines and antibodies. On day 5 after activation C2GlcNAcT-I (A) and FucT-VII mRNA expression (B) expression normalized to HPRT mRNA expression were determined ($n \geq 3$; mean ± SD. * $p<0.05$ vs. Th0, **$p<0.01$ vs. Th0, ***$p<0.001$ vs. Th0; unpaired t-test). C) Naive $CD4^+$ T cells from wildtype (WT) and T-$bet^{-/-}$ mice were activated by anti-CD3/anti-CD28 stimulation under Th1 polarizing conditions (IL-2 + IL-12 + IFNγ + anti-IL-4). P-lig expression and FucT-VII and C2GlcNAcT-I mRNA expression was analyzed on day 3 after activation ($n = 3$, mean ± SD).

Figure 6: Blockade of IL-2 abrogates FucT-VII and E-lig induction

Naive $CD4^+$ T cells were stimulated by anti-CD3/anti-CD28 for 3 days under (A) Th0 (anti-IL-12 + anti-IFNγ + anti-IL-4) or (B) Th1 (IL-12 + IFNγ + anti-IL4) polarizing conditions in the presence or absence of additional IL-2. IL-2 treated T cells were further expanded in the presence of IL-2 while T cells cultured without exogenous IL-2 were cultured in the presence of anti-IL-2. IL-7 was added as indicated in addition to anti-IL-2. 24 h later P-lig, E-lig expression as well as C2GlcNAcT-I and

FucT-VII mRNA relative to 18s RNA expression was determined. (n ≥ 3; mean ± SD). * p<0.05, n.s. not significant (Wilcoxon matched pairs test).

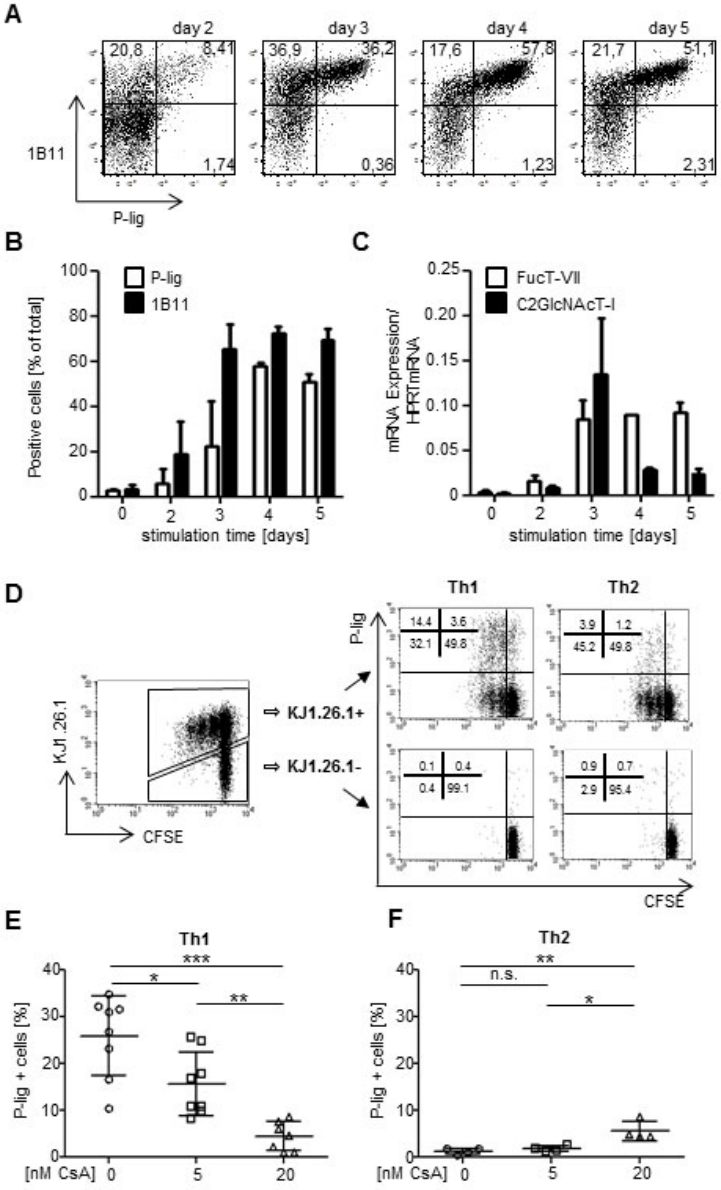

Figure 1

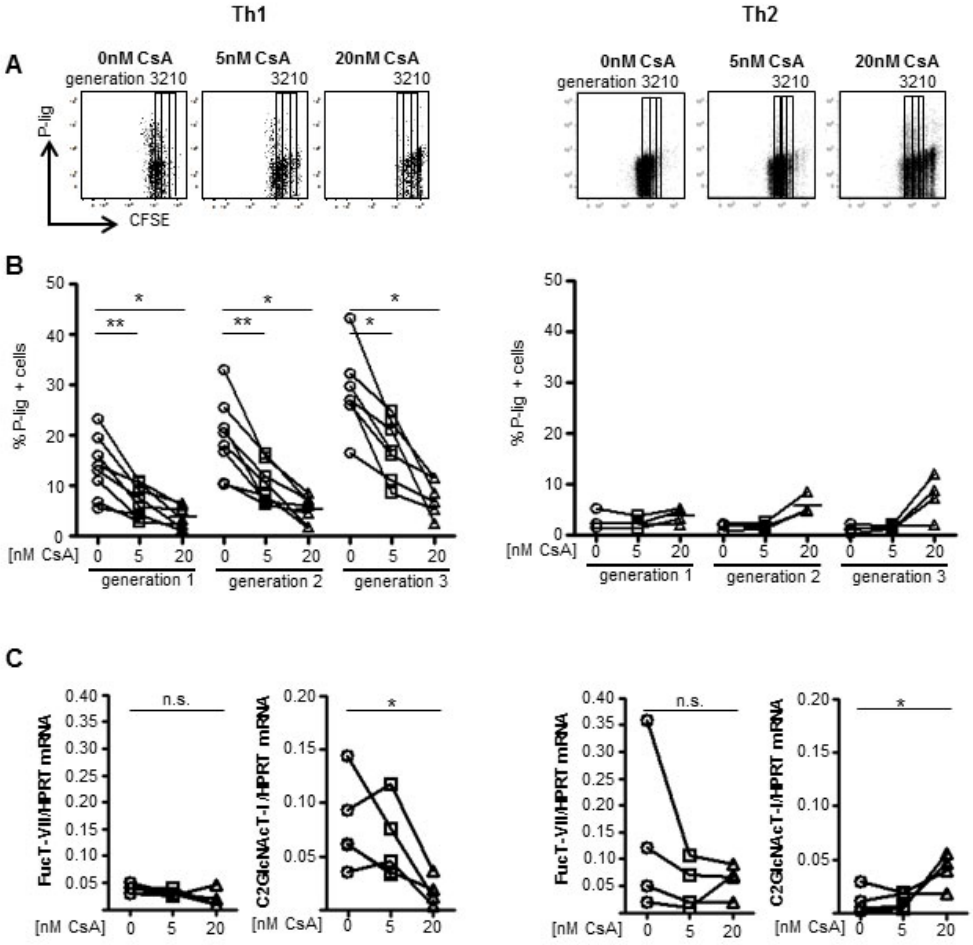

Figure 2

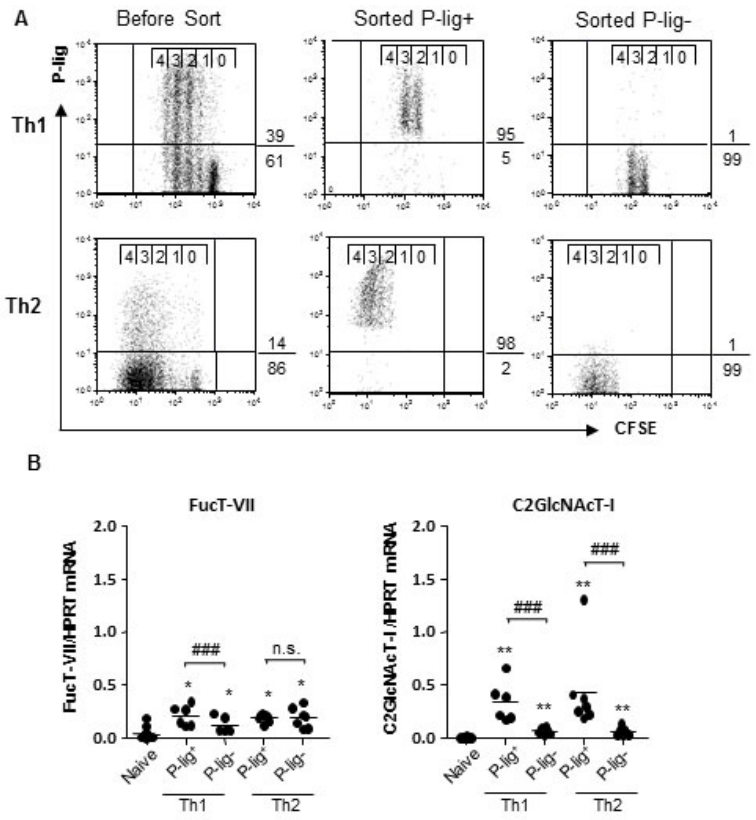

Figure 3

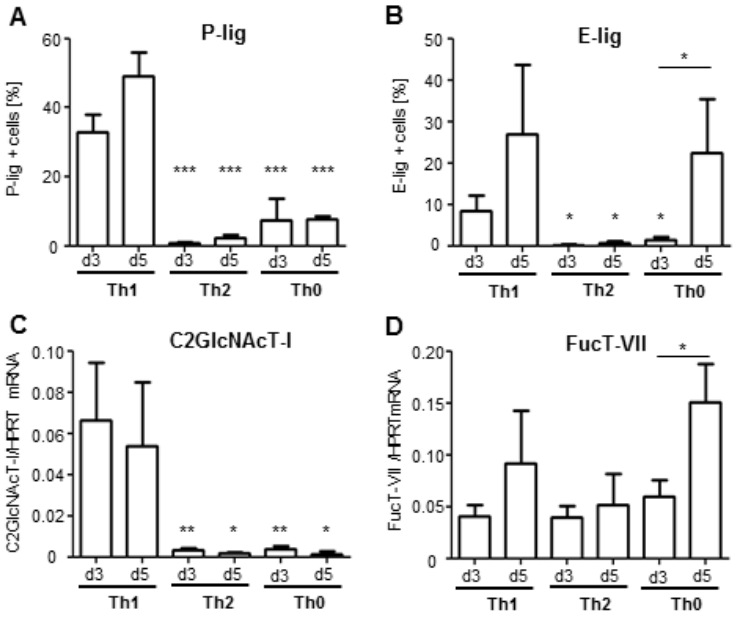

Figure 4

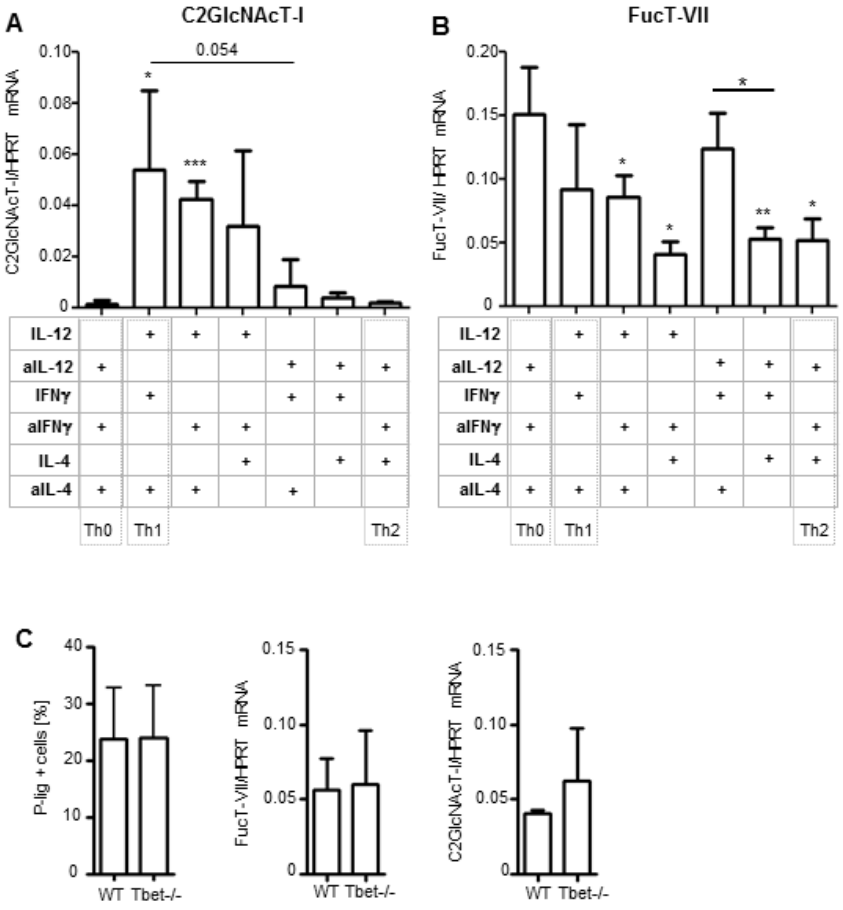

Figure 5

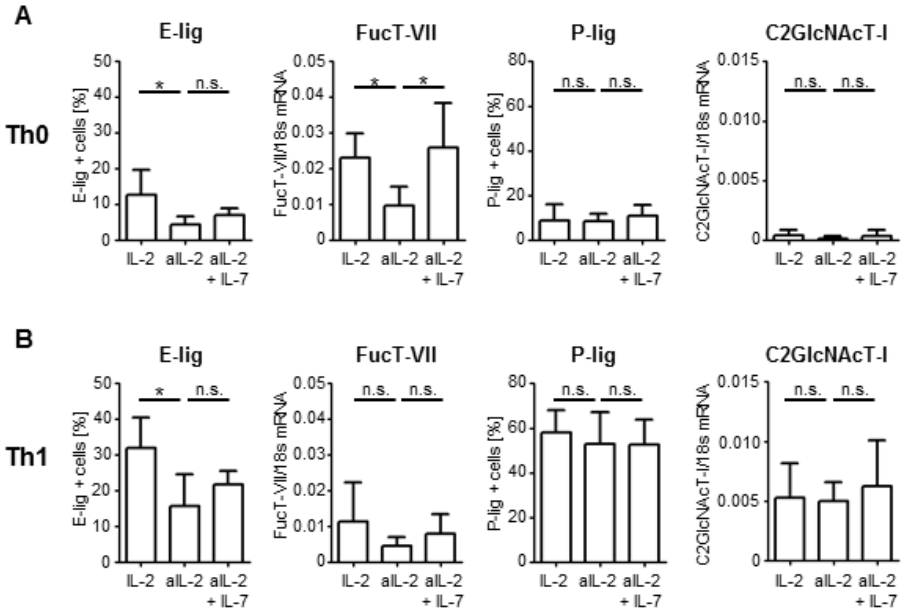

Figure 6