

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

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Introduction

P-selectin and E-selectin ligands (P-lig and E-lig) are involved in the recruitment of leucocytes into inflamed tissues. Myeloid cells express the ligands constitutively but lymphocytes show a restricted expression on subsets of memory/effector cells.^{1,2} Selectin-binding epitopes are

Summary

Ligands for E-selectin and P-selectin (E-lig and P-lig) are induced on CD4⁺ T cells upon differentiation into effector T cells. Glycosyltransferases, especially α 1,3-fucosyltransferase VII (FucT-VII) and core 2 β 1,6-*N*-acetyl-glycosaminyltransferase I (C2GlcNAcT-I), are critical for their synthesis. We here analysed the signals that control the expression of E-lig, P-lig and mRNA coding for FucT-VII and C2GlcNAcT-I. In line with previous reports, we found that P-lig expression correlates with the regulation of C2GlcNAcT-I, whereas 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 up-regulation under permissive (T helper type 1) conditions was strongly reduced by cyclosporin A (CsA), suggesting the involvement of T-cell receptor-dependent, calcineurin/NFAT-dependent signals in combination with interleukin-12 (IL-12) -mediated signals in the regulation of C2GlcNAcT-I. In contrast, expression of FucT-VII mRNA was not significantly inhibited by CsA. Interleukin-4 inhibited the expression of FucT-VII but IL-2 and IL-7 were found to support induction of FucT-VII and E-lig. E-selectin, P-selectin and their ligands initially appeared to have rather overlapping functions. These findings however, unravel striking differences in the regulation of 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 T-cell receptor signal in combination with inflammatory cytokines in case of C2GlcNAcT-I.

Keywords: fucosyltransferase; interleukin-2; P-selectin ligand; T-cell activation.

generated after post-translational modification of protein carriers such as P-selectin glycoprotein ligand-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 β 1,6-*N*-acetyl-glycosaminyltransferase I (C2GlcNAcT-I) are particularly

Abbreviations: APC, allophycocyanin; C2GlcNAcT-I, core 2 β 1,6-glycosaminyltransferase I; CFSE, 5-(and 6-)carboxy-fluorescein diacetate succinimidyl ester; CsA, cyclosporin A; E-lig, E-selectin ligand; FucT-VII, fucosyltransferase VII; IFN- γ , interferon- γ ; IL-2, interleukin-2; mAb, monoclonal antibody; MEK, mitogen-activated protein kinase kinase; NFAT, nuclear factor of activated T cells; PE, phycoerythrin; P-lig, P-selectin ligand; STAT4, signal transducer and activator of transcription 4; Th1, T helper type 1

important for the generation of selectin-binding epitopes in lymphocytes because deletion of either one of these transferases results in the almost complete lack of E-lig and P-lig 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-lig are induced in CD4⁺ T cells concomitant with the T helper type 1 (Th1) differentiation programme.⁶ Studies using human T cells suggested that FucT-VII is induced by interleukin-12 (IL-12) and counter-regulated by IL-4.¹ In contrast, in the murine system, Lim *et al.*⁷ and White *et al.*⁸ showed by using T cells that were deficient for signal transducer and activator of transcription (STAT4), which have impaired intracellular IL-12 signalling, that IL-12 induces C2GlcNAcT-I expression but not FucT-VII expression. T-box expressed in T cells (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} Taken together, 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 T-cell receptor (TCR) signalling on FucT-VII induction was investigated by Barry *et al.*,¹¹ who showed that FucT-VII induction in a PMA-responsive human T-cell line was reduced by combined treatment with the p38 mitogen-activated protein kinases inhibitor SB203580 and the mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor PD98059. 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 nuclear factor of activated T cells (NFAT) proteins, which activate a number of target genes after nuclear translocation. To investigate the role of this signalling 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-lig 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 an unrecognized role of

common γ -chain cytokines in the regulation of FucT-VII in activated T cells and suggest a functional dichotomy among the ligands for E-selectin and P-selectin in that FucT-VII-dominated synthesis of E-lig which is predominantly driven by homeostatic cytokines whereas 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 gift from D.Y.Loh (Washington University School of Medicine, St Louis, MO) and T-bet^{-/-} mice were a gift from A. Radbruch (Deutsches Rheuma-Forschungszentrum, Berlin, 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 Rheuma-Forschungszentrum (Berlin, Germany): anti-CD3 (145-2C11), anti-CD28 (37.51), anti-IL-4 (11B11), anti-IL-12 (C17.8), anti-interferon- γ (anti-IFN- γ ; AN18.17.26) and anti-IL-2 (S4B6). Anti-CD4-FITC (L3T4), anti-CD62L-phycoerythrin (PE) (MEL-14), anti-CD25-allophycocyanin (APC) (PC61) and anti-CD90-Peridinin chlorophyll protein (Ox-7) were purchased from BD Biosciences (Franklin Lakes, NJ); anti-T-bet-APC (eBio4B10), anti-CD4-Alexafluor450 (L3T4), anti-CD62L-efluor450 (MEL-14) and isotype controls were from eBioscience (Hatfield, UK); anti-CD43 (activated Glycoform)-PE-Cy7 (1B11) were from BioLegend (San Diego, CA); and PE-conjugated F(ab')₂ donkey anti-human IgG antibody was from Jackson Immuno Research (West Grove, PA). All microbeads were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany).

Cell culture was set up in complete RPMI-1640 (Gibco Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (Sigma-Aldrich, Taufkirchen, Germany), 5 mM HEPES, penicillin and streptomycin, 1 mM sodium pyruvate (all from Biochrom AG, Berlin, Germany), and 10 μ M 2-mercaptoethanol (Gibco Invitrogen). Recombinant murine IL-2, IL-7, IL-12, IFN- γ , IL-4 and E-selectin-human IgG chimeric protein were 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 and pelvic and mesenteric lymph nodes were pooled and stained with anti-CD4-FITC monoclonal antibody (mAb) and anti-FITC multisort magnetic activated cell sorting (MACS) beads. Cells were sorted using an AutoMACS (Miltenyi Biotech, Bergisch-Gladbach, Germany) reaching a purity of at least 95%. After release of multisort beads according to the manufacturer's 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 ovalbumin (OVA) TCR^{tg} T cells were cultured with antigen-presenting cells at a ratio of 1 : 4 in the presence of 0.5 μ M OVA_{323–339} peptide (Biochemistry Department, Charité Universitätsmedizin, Berlin, Germany). For polyclonal activation, sorted naive T cells were cultured on plates coated with anti-CD3 and anti-CD28 mAbs at 1×10^6 cells/ml. For Th1 polarizing conditions cultures were supplemented with recombinant murine IL-12 at 5 ng/ml, IFN- γ at 20 ng/ml and neutralizing anti-IL-4 mAb at 5 μ g/ml. For Th2 polarizing conditions recombinant murine IL-4 at 30 ng/ml, neutralizing anti-IL-12 mAb at 5 μ g/ml and neutralizing anti-IFN- γ mAb at 5 μ g/ml were added to the culture. For Th0 conditions neutralizing anti-IL-12 mAb, anti-IFN- γ and anti-IL-4 mAb were added to the culture. Either IL-2 or IL-7 was added at 10 ng/ml as indicated. For CsA treatment T cells were incubated for 20 min at 37° in 5% CO₂ with CsA (Sigma-Aldrich) before cytokines, antibodies and stimulus were added.

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

Naive CD4⁺ T cells were washed with PBS before labelling and resuspended at 1×10^7 cells/ml in PBS containing 5 μ M CFSE (Molecular Probes, Gibco Invitrogen). After incubation for 2.5 min at room temperature the reaction was stopped by adding RPMI-1640 with 10% fetal calf serum.

Cytometric analysis and FACS sorting

For cytometric analysis cells were stained with the respective antibodies for 20 min at 4° in the dark. The P-lig and E-lig were detected with the P-selectin- and E-selectin-human IgG chimeric proteins and PE-conjugated anti-human IgG antibody F(ab')₂ as a secondary reagent.

Staining was performed in Ca²⁺-containing Hanks' balanced salt solution buffer supplemented with 10 mM HEPES as previously described.¹³ A FACSCalibur, LSR II or LSRFortessa (all BD Biosciences) and the CELLQUEST PRO (BD Biosciences) or FLOWJO (Tree Star, Ashland, OR) software was 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 fractions. Dead cells were excluded according to propidium iodide or 4,6-diamidino-2-phenylindole 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 FACS Aria or a FACS Diva (all BD Biosciences).

Quantitative PCR

Total RNA was isolated with an RNeasy Mini Kit and QiaShredder (Qiagen, Hilden, Germany) and DNA was removed with an 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 described¹³ using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) or by using the platinum SYBR Green qPCR Super-Mix-UDG (Gibco Invitrogen) and the same primer pairs and the Mx3000P or Mx3005P qPCR system (Agilent Technologies, Santa Clara, CA).

Statistics

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

Results

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

As known, activation of naive CD4⁺ T cells under permissive (Th1) conditions leads to 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 slowly up-regulated and reach high levels only at or from day 3 (Fig. 1a–c).

This raises the question whether T-cell signals induce FucT-VII and C2GlcNAcT-I directly or whether the regulation rather occurs indirectly, e.g. via cytokines or

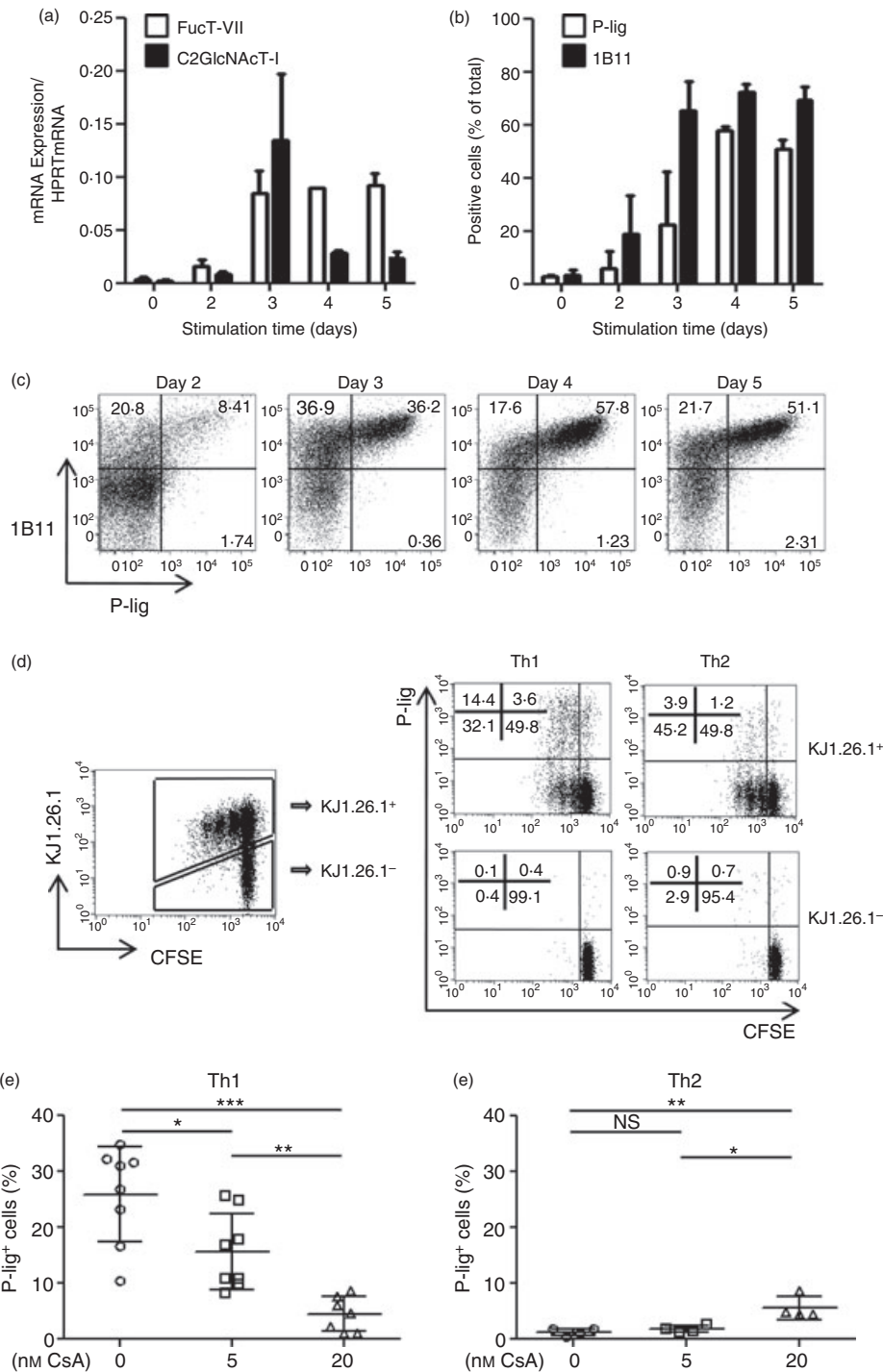


Figure 1. T-cell receptor signalling is required for induction of P-selectin ligation (P-lig). Naive T cells from BALB/c mice were activated by anti-CD3/anti-CD28 stimulation under T helper type 1 (Th1) conditions [(interleukin-2 (IL-2) + IL-12 + interferon- γ (IFN γ) + anti-IL-4). 1B11 and P-lig expression (staining example: a; summary: b) and fucosyltransferase VII (FucT-VII) and core 2 β 1,6-*N*-acetyl-glycosaminyltransferase I (C2-GlcNAcT-I) mRNA expression (c) were determined at indicated time-points of culture. Carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labelled naive CD4⁺ T cells from DO11.10 mice were activated in the presence of ovalbumin₃₂₃₋₃₃₉ peptide, allophycocyanin and Th1 polarizing cytokines (IL-12 + IFN- γ + anti-IL-4) or Th2 polarizing conditions (anti-IFN- γ + anti-IL-12 + IL-4). Three days after activation CD4⁺ cells were stained for expression of the transgenic ovalbumin-specific TCR using the clonotype-specific antibody KJ1.26.1 (d). Proliferation determined by loss of CFSE and P-lig induction was analysed for transgenic and non-transgenic T cells. Cyclosporin A (CsA) was added to polyclonally activated Th1 (IL-12 + IFN- γ + anti-IL-4) and Th2 cultures (anti-IFN- γ + 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 \pm SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; unpaired *t*-test).

cytokine receptors. First, we exploited the heterogeneous expression of the transgenic TCR in DO11.10 mice to analyse whether P-lig induction after activation of naive CD4⁺ T cells depends on a cell-intrinsic TCR signal or on changes in the milieu. As shown in Fig. 1(d), P-lig induction was observed only on T cells expressing the transgenic OVA-specific TCR (KJ1.26.1⁺). Second, we used 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 (Fig. 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 (Fig. 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 analysed in individual generations detected by loss of CFSE (Fig. 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 (Fig. 2b).

Hence, 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 (Fig. 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 (Fig. 2c). 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 conditions was found to correlate to C2GlcNAcT-I, but not FucT-VII 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 Figs 1 and 2, only a fraction of T cells expresses P-lig in Th1 cultures and, an even smaller fraction in Th2 cultures. To analyse 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. The Th1 cells were sorted from day 3 cultures, a time-point with intermediate levels of P-lig⁺ cells (see following Fig. 4) allowing purification of P-lig⁺ and P-lig⁻ T-cell fractions. 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 because of 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⁻ fractions. To exclude any bias caused by 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 (Fig. 3a). FucT-VII and C2GlcNAcT-I mRNA was up-regulated in both P-lig⁺ and P-lig⁻ fractions compared with naive T cells (Fig. 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 more strongly 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. Interleukin-2 was added to all cultures. In addition to P-lig, we also determined E-lig expression 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 (Fig. 4a,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 (Fig. 4b,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 (Fig. 4d). These data confirm that C2GlcNAcT-I determines the 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, IFN- γ and IL-4 or blocking antibodies. Interferon- γ 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

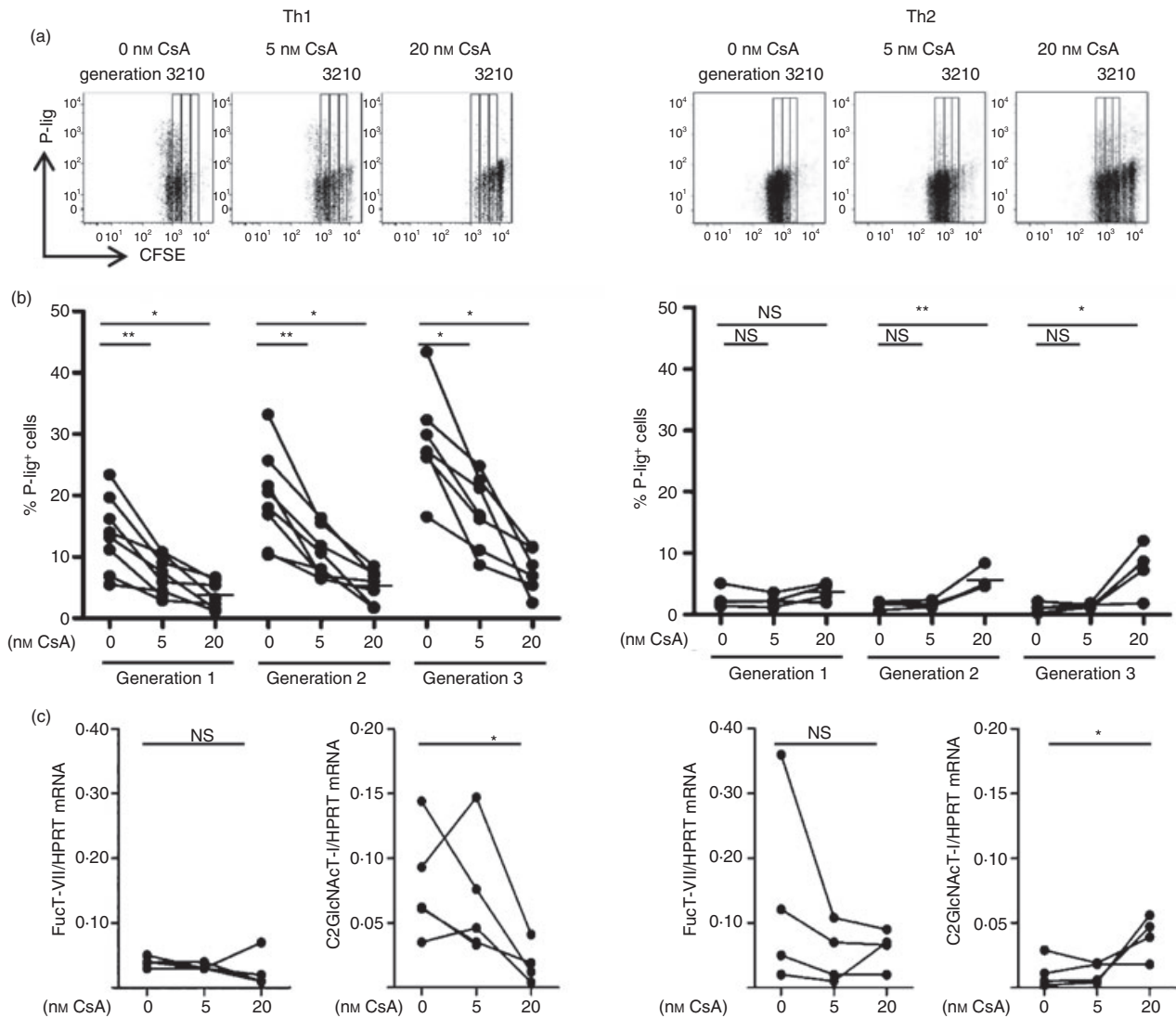


Figure 2. Differential effect of cyclosporin A (CsA) on P-selectin ligand (P-lig) induction under T helper type 1 (Th1) and Th2 polarizing conditions. Carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labelled naive CD4⁺ T cells from BALB/c mice were activated by anti-CD3/anti-CD28 stimulation under Th1 [interleukin-12 (IL-12) + interferon- γ (IFN- γ) + anti-IL-4] or Th2 polarizing (anti-IL-12 + anti-IFN- γ + 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 (right panel) cultures. In (b) the summary of P-lig expression analysed in generations 1–3 of Th1-polarized and Th2-polarized cells is shown (Th1: $n \geq 6$; Th2: $n = 4$). In (c) fucosyltransferase VII (FucT-VII) and core 2 β 1,6-*N*-acetyl-glycosaminyltransferase I (C2GlcNAcT-I) mRNA expression for FACS-sorted cells of generation 3 ($n \geq 4$) (mean \pm SD * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant; unpaired *t*-test).

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

T-bet is induced by IFN- γ and IL-12 signalling.¹⁴ Since previous studies suggested that T-bet is involved in

C2GlcNAcT-I induction^{9,10} we analysed 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 expression of P-lig, C2GlcNAcT-I and FucT-VII in T-bet-deficient cells compared with wild-type controls (Fig. 5c) at this early time-point, suggesting that T-bet induced by T-cell activation or IFN- γ is not involved in the primary steps of the IL-12-dependent induction of C2GlcNAcT-I.

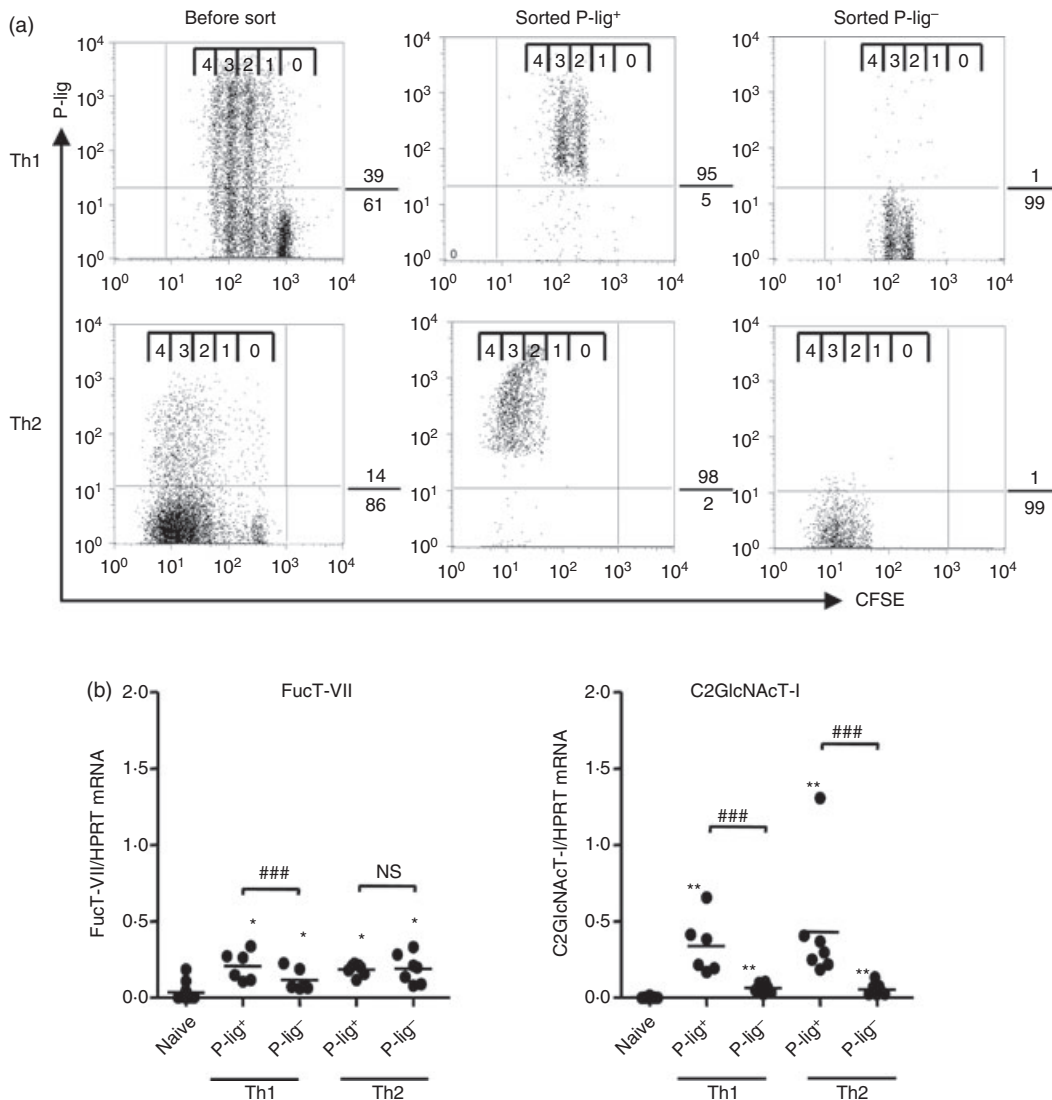


Figure 3. Differential expression of core 2 β 1,6-*N*-acetyl-glycosaminyltransferase I (C2GlcNAcT-I) mRNA in P-selectin ligand-positive (P-lig⁺) and P-lig⁻ cell fraction from T helper type 1 (Th1) and Th2 cultures. Carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labelled, naive CD4⁺ T cells were activated by anti-CD3/anti-CD28 stimulation under Th1 [interleukin-2 (IL-2) + IL-12 + interferon- γ (IFN- γ) + anti-IL-4] or Th2 (IL-2 + anti-IL-12 + anti-IFN- γ + 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). Messenger RNA expression of fucosyltransferase VII (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 ** $P < 0.01$ versus naive T cells, Mann-Whitney U -test; ### $P < 0.001$, paired t -test.

Blockade of IL-2 inhibits FucT-VII expression

To confirm the hitherto unreported 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 either exogenous IL-2 or a neutralizing anti-IL-2 mAb. Blockade of IL-2 reduced E-lig as well as FucT-VII up-regulation in T cells cultured under Th0 conditions (Fig. 6a). Interleukin-7, which also signals via the common γ -chain of the IL-2 receptor, could restore FucT-VII

mRNA expression (Fig. 5a). Expression of P-lig and C2GlcNAcT-I, which is low under these conditions, was unaffected by IL-2 blockade (Fig. 5a). Under Th1 conditions, IL-2 blockade also resulted in reduced expression of E-lig and a tendency toward reduced FucT-VII (Fig. 6b). Interleukin-7 could at least partially rescue the effect on E-lig expression, whereas IL-12 and IFN- γ present under Th1 conditions were obviously unable to do so (Fig. 6b). P-lig as well as C2GlcNAcT-I mRNA expression were unaffected by IL-2 blockade.

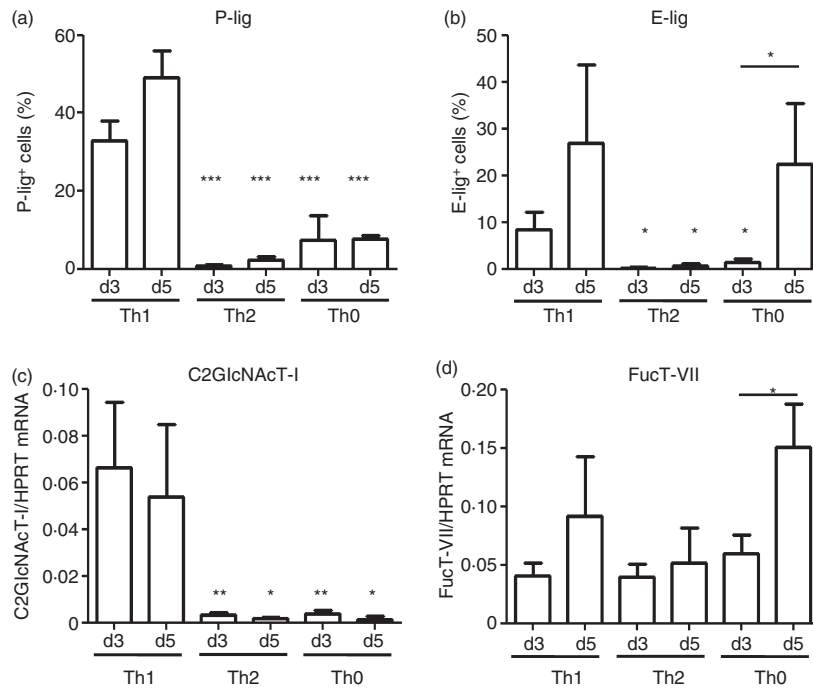


Figure 4. Fucosyltransferase VII (FucT-VII) and E-selectin ligand (E-lig) expression is promoted under T helper type 0 (Th0) conditions in the absence of T-cell receptor (TCR) stimulation. Naive CD4⁺ T cells were activated by anti-CD3/anti-CD28 stimulation under Th1 [interleukin-2 (IL-2) + IL-12 + interferon- γ (IFN- γ) + anti-IL-4], Th2 (IL-2 + anti-IL-12 + anti-IFN- γ + IL-4) or Th0 (IL-2 + anti-IL-12 + anti-IFN- γ + anti-IL-4) polarizing conditions. P-lig (a) and E-lig (b) expression as well as core 2 β 1,6-*N*-acetyl-glycosaminyltransferase I (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 \pm SD). *** $P < 0.001$ versus Th1 same day; ** $P < 0.01$ versus Th1 same day; * $P < 0.05$ versus Th1 same day; unpaired *t*-test.

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, whereas 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 with low P-lig induction. However, residual expression or low induction of FucT-VII still seems to be required for P-lig synthesis because Th1 cells from FucT-VII^{-/-} mice lack P-lig expression and show impaired recruitment into acute inflamed delayed-type hypersensitivity 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, whereas 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. Interleukin-4 had no direct suppressive effect on C2GlcNAcT-I expression and in contrast to other studies, we found no major inducing effect of IFN- γ and T-bet on the expression of P-lig and C2GlcNAcT-I.^{9,10} However, in the study by Underhill *et al.*⁹ the differences between wild-type and T-bet^{-/-} T cells were most prominent after prolonged culture. As we only analysed early time-points our data do not preclude that T-bet acts at later time-points for instance by stabilizing 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

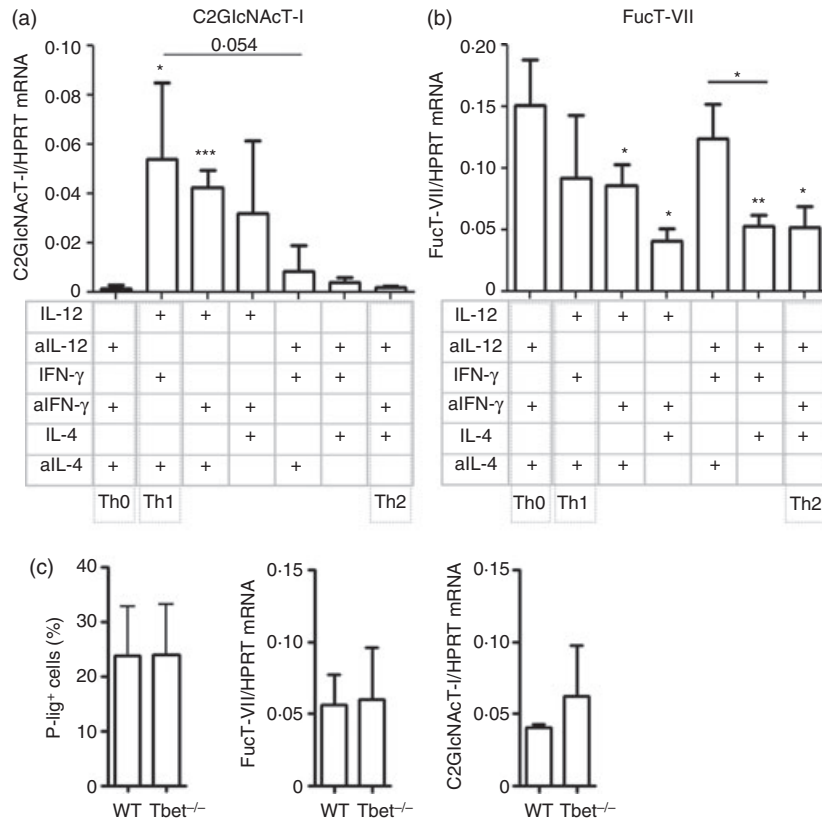


Figure 5. Core 2 β 1,6-*N*-acetyl-glycosaminyltransferase I (C2GlcNAcT-I) is induced by interleukin-12 (IL-12), whereas fucosyltransferase VII (FucT-VII) is induced in the presence of IL-2 and suppressed by IL-4 and IL-12. Naive T cells were activated by anti-CD3/anti-CD28 stimulation in the presence 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 \pm SD). * $P < 0.05$ versus Th0; ** $P < 0.01$ versus Th0; *** $P < 0.001$ versus Th0; unpaired *t*-test. (c) Naive CD4⁺ T cells from wild-type (WT) and Tbet^{-/-} mice were activated by anti-CD3/anti-CD28 stimulation under Thelper type 1 (Th1) polarizing conditions [IL-2 + IL-12 + interferon- γ (IFN- γ) + anti-IL-4]. P-selectin ligand (P-lig) expression and FucT-VII and C2GlcNAcT-I mRNA expression were analysed on day 3 after activation ($n = 3$, mean \pm SD).

observed that IL-12 in the absence of IFN- γ suppressed FucT-VII expression, though less strongly than IL-4.¹ However, addition of IFN- γ 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 *et al.* study were performed with total CD4⁺ T cells and without blockade of IFN- γ , which rather reflects Th1 conditions, both studies concur that Th1 conditions support high FucT-VII induction.

Interleukin-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 γ -chain cytokine¹⁶ which suggests that promiscuous 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 signalling, in particular, p38-dependent and Ras-Raf-dependent pathways were shown to control FucT-VII induction.^{11,12} We found that blocking of calcineurin-dependent pathways of T-cell activation by CsA inhibited C2GlcNAcT-I but not FucT-VII mRNA induction. This is surprising as 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, act at later time-points and that Ras-Raf-dependent pathways, possibly less affected by the CsA treatment, might control the early TCR-dependent FucT-VII induc-

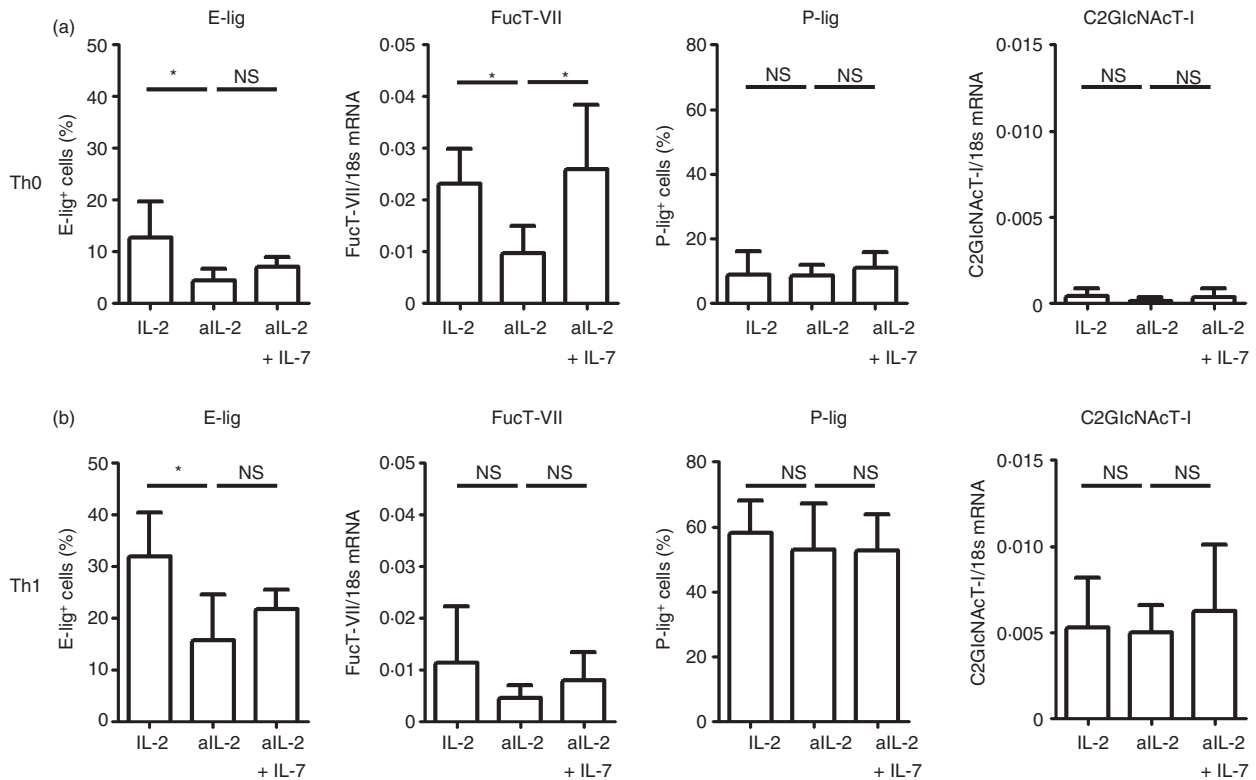


Figure 6. Blockade of interleukin-2 (IL-2) abrogates fucosyltransferase VII (FucT-VII) and E-selectin ligand (E-lig) induction. Naive CD4⁺ T cells were stimulated by anti-CD3/anti-CD28 for 3 days under (a) T helper type 0 (Th0) [anti-IL-12 + anti-interferon- γ (IFN- γ) + anti-IL-4] or (b) Th1 (IL-12 + IFN γ + anti-IL4) polarizing conditions in the presence or absence of additional IL-2. The IL-2 treated T cells were further expanded in the presence of IL-2 whereas T cells cultured without exogenous IL-2 were cultured in the presence of anti-IL-2. Interleukin-7 was added as indicated in addition to anti-IL-2. Twenty-four hours later P-lig and E-lig expression as well as core 2 β 1,6-*N*-acetyl-glycosaminyltransferase I (C2GlcNAcT-I) and FucT-VII mRNA relative to 18s RNA expression was determined. ($n \geq 3$; mean \pm SD). * $P < 0.05$; NS, not significant (Wilcoxon matched pairs test).

tion. Alternatively, minute amounts of IL-2 produced even in the presence of CsA might be sufficient to induce FucT-VII.

In a previous study, we showed that FucT-VII induction in naive 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 or E-lig, suggests 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 the possibility that the effect of CsA is due to impaired availability of IL-2, this appears unlikely because 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 only observed 48–72 hr after activation.

Hence, 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-12 receptor β 2 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 signalling pathways, i.e. NFAT-dependent 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 cannot be explained by changes in IL-4 signalling due to CsA treatment because IL-4 did not actively suppress C2GlcNAcT-I mRNA expression (Fig. 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 because *in vivo* the majority of IL-4-producing CD4⁺ T cells coexpress P-lig.¹⁸

Taken together, our data show striking differences between 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. Whereas IL-12 and a strong TCR signal up-regulate 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 γ -chain of the IL-2 receptor, suggest that STAT5 signalling is involved in transcriptional activation of *fucosyltransferase 7*.

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Disclosures

The authors do not have any conflicts of interests.

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 $\alpha 1$, 3-fucosyltransferase VII gene expression. *J Exp Med* 1998; **188**: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**:3193–201.
- 3 Lowe JB. Glycan-dependent leukocyte adhesion and recruitment in inflammation. *Curr Opin Cell Biol* 2003; **15**: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**:601–14.
- 5 Snapp KR, Heitzig CE, Ellies LG, Marth JD, Kansas GS. Differential requirements for the O-linked branching enzyme core 2 β 1-6-N-glucosaminyltransferase in biosynthesis of ligands for E-selectin and P-selectin. *Blood* 2001; **97**: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**: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 β -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**: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**: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**: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**:1771–8.
- 12 Zisoulis DG, Kansas GS. H-Ras and phosphoinositide 3-kinase cooperate to induce α (1,3)-fucosyltransferase VII expression in Jurkat T cells. *J Biol Chem* 2004; **279**:39495–504.
- 13 Syrbe U, Jennrich S, Schottelius A, Richter A, Radbruch A, Hamann A. Differential regulation of P-selectin ligand expression in naive 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- γ and interleukin-12. *Immunity* 2009; **30**:673–83.
- 15 Doebeis C, Siegmund K, Lodenkemper 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**: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**: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**: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**:3070–81.