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Core 2 ß1,6-N-acetylglucosaminyltransferase-I, crucial for P-selectin ligand expression is controlled by a distal enhancer regulated by STAT4 and T-bet in CD4+ T helper cells 1

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ABSTRACT

P-selectin ligands (P-ligs) support the recruitment of lymphocytes into inflamed tissues. Binding to P-selectin is mediated by oligosaccharide groups synthesized by means of several glycosyltransferases including core 2 ß1,6-*N*-acetylglucosaminyltransferase-I (C2GlcNAcT-I), encoded by the gene *Gcnt1*. Using *Gcnt1*$^{-/-}$ Th1 cells, we show that C2GlcNAcT-I is crucial for inflammatory T cell homing *in vivo*. To understand the molecular regulation of *Gcnt1* in CD4^{+} T helper cells, we performed ChIP-on-chip experiments across the *Gcnt1* locus assessing the chromatin structure in P-lig-expressing *versus* non-expressing CD4^{+} T cells. This identified a distal region about 20 kb upstream of the promoter where the presence of a H3K27me3 mark correlated with *Gcnt1* repression. This region possessed IL-12-dependent enhancer activity in reporter assays, in accordance with preferential IL-12-dependent induction of *Gcnt1 in vitro*. STAT4 and T-bet cooperated in control of the enhancer activity. Deficiency in either one resulted in drastically reduced Gcnt1 mRNA expression in differentiated Th1 cells. While both STAT4 and T-bet were bound to the enhancer early after activation only T-bet binding persisted throughout the expansion phase after TCR signal cessation. This suggests sequential action of STAT4 and T-bet at the enhancer.

In summary, we show that *Gcnt1* transcription and subsequent P-lig induction in Th1 cells is governed by binding of STAT4 and T-bet to a distal enhancer and further regulated by epigenetic marks such as H3K27me3.

1. Introduction

E- and P-selectin ligands (E- and P-ligs) are an essential part of the homing system of T cells to the skin, however, especially P-ligs also promote leukocyte recruitment into inflamed sites outside the skin (Ley, 2003). Most P-selectin-binding epitopes are displayed on the carrier protein P-selectin glycoprotein 1 (PSGL-1) (Hirata et al., 2000) and consist of sialyl Lewis X related oligosaccharide structures (Lowe, 2003). Several glycosyltransferases are involved in the synthesis of these post-translational modifications including fucosyltransferase (FucT)-VII, the *O*-linked branching enzyme core-2-ß1,6-*N*-acetylglucosaminyltransferase-I (C2GlcNAcT-I), a ß1,4-galactosyltransferase-I, and at least one sialyltransferase of the ST3Gal family (Ley, 2003).

In T cells, expression of E- and P-ligs is inducible upon activation and subsequent polarization into effector/memory T cells. It corresponds to induction of FucT-VII and C2GlcNAcT-I mRNA (Ley, 2003). While FucT-VII is essential for the synthesis of both E- and P-ligs (Ley, 2003; Weninger et al., 2000) C2GlcNAcT-I primarily promotes P-lig generation (Snapp et al., 2001). As such, deficiency in FucT-VII, encoded by the gene *Fut7*, results in a dramatic reduction of E- and P-lig on activated T cells (Ellies et al., 1998; Weninger et al.,

Abbreviations: E-lig, E-selectin ligand; P-lig, P-selectin ligand; FucT, fucosyltransferase; C2GlcNAcT-I, core 2 (beta) 1,6-glycosaminyltransferase I; OVA, ovalbumin; CFSE, 5-(and 6-)carboxy-fluorescein diacetate succinimidyl ester; DTH, delayed type hypersensitivity; IFA, incomplete Freund's adjuvant.

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2000). In contrast, deletion of *Gcnt1*, the gene encoding C2GlcNAcT-I, abrogates P-lig, but not E-lig, expression in activated T cells (Snapp et al., 2001). C2GlcNAcT-I is also required for P-lig synthesis in neutrophils and disrupted recruitment into inflamed tissues has been described for *Gcnt1*$^{-/-}$ neutrophils (Snapp et al., 2001; Sperandio et al., 2001).

While *in vivo* E- and P-ligs are expressed on both Th1 and Th2 effector cells, as well as on subsets of T regulatory cells (Huehn et al., 2004; Kretschmer et al., 2004; Teraki and Picker, 1997), induction *in vitro* is primarily observed under Th1 polarizing conditions which largely depends on IL-12. This IL-12-dependent induction of *Gcnt1* transcription in Th1 cells is controlled by STAT4, a downstream signaling molecule of IL-12 (Lim et al., 2001; White et al., 2001). T-bet, encoded by the *Tbx21* gene, which is also at least partially induced by IL-12 (Schulz et al., 2009), might also play a role in *Gcnt1* induction in Th1 cells. Supporting that notion, two studies reported impaired P-selectin expression or inflammatory homing in T-bet-deficient T cells (Lord et al., 2005; Underhill et al., 2005). In contrast, we observed no difference in *Gcnt1* expression in *in vitro* activated *Tbx21*$^{-/-}$ Th1 cells during TCR stimulation (Schroeter et al., 2012).

According to our previous data, P-ligs become permanently expressed at least on a fraction of *in vitro* generated Th1 cells generating a stable skin- and inflammation-seeking homing T cell subset (Jennrich et al., 2007). As epigenetic signatures, such as DNA methylation and histone modifications, control gene accessibility and commitment to a particular T effector cell subset (Kanno et al., 2012) such mechanisms might also be involved in control of permanent P-lig expression. In support of this view, P-lig induction not only requires Calcineurin-dependent TCR signals (Schroeter et al., 2012) but also progression through the cell cycle, a window for chromatin modifications (Syrbe et al., 2004). Mono/di/trimethylation of Lysine 4 on histone 3, H3K4me1/2/3, represents a permissive active chromatin configuration while trimethylation at Lysine 27 on histone 3, H3K27me3, corresponds to a repressed state of transcription (Heintzman et al., 2007; Wei et al., 2009). The simultaneous presence of histone modifications associated with both gene activation and repression is defined as a poised (bivalent) chromatin that, in principle, is accessible for transcription factors (Bernstein et al., 2006). Differential epigenetic marks often mark regulatory elements (Heintzman et al., 2009); hence, their detection may help to elucidate the molecular regulation of genes.

In this study, we first show the requirement of C2GlcNAcT-I for inflammatory homing of Th1 cells to the skin. Furthermore, we aimed to understand the molecular regulation of P-lig induction and long-term expression in T cells and therefore searched for regulatory regions within the *Gcnt1* gene. Apart from the promoter region, we identified a distal region about 20 kb upstream of the *Gcnt1* gene where the presence of a distinctive H3K27me3 mark correlated with *Gcnt1* repression in CD4$^+$ T cells. This region elicited STAT4-dependent enhancer activity in reporter gene assays and bound STAT4 as well as T-bet during Th1 differentiation. While STAT4 binding peaked early, persistent T-bet binding throughout the expansion phase suggests that STAT4 and T-bet sequentially act on the distal enhancer to drive *Gcnt1* expression in Th1 cells.

2. Materials and methods

2.1. Mice

BALB/c and C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). *Gcnt1*$^{-/-}$ mice were generated in the J. Marth laboratory (University of California) (Ellies et al., 1998) and provided by M. Sperandio (Ludwig-Maximilians-University, Munich, Germany). *Tbx21*$^{-/-}$ mice were a kind gift from M. Löhning (DRFZ, Berlin, Germany). Ovalbumin(OVA)-transgenic OT-II, *Fut4+7*$^{-/-}$ and *Stat4*$^{-/-}$ mice were acquired from the Bundesinstitut für Risikobewertung (Berlin, Germany). All animal experiments were performed in accordance with institutional, state and federal guidelines.

2.2. Antibodies, recombinant cytokines, and magnetic beads

The following antibodies (clones) were produced in-house at the Deutsches Rheuma-Forschungszentrum (Berlin, Germany): anti-CD3 (145-2C11), anti-CD28 (37.51), anti-IL-4 (11B11), anti-IL12 (C17.8) and anti-IFNγ (AN18.726). 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-CD44-PE-Cy7 (IM7) from eBioscience (Hatfield, UK), and PE-conjugated F(abí)$_2$ donkey anti-human IgG antibody from Jackson ImmunoResearch (West Grove, USA). All magnetic activated cell sorting (MACS) microbeads were obtained and used as described by the manufacturer Miltenyi Biotec (Bergisch Gladbach, Germany). Recombinant murine IL-2, IL-12, IFNγ, IL-4 and P-selectin-human IgG chimeric protein were purchased from R&D Systems (Wiesbaden, Germany).

2.3. Isolation, cell purification, and cell culture

Naive CD4$^+$ T cells were purified from pooled spleens, peripheral, and mesenteric lymph nodes, after erythrocyte lysis, by depletion of CD25$^+$ cells using anti-CD25-APC and anti-APC beads with subsequent enrichment of CD4$^+$ T cells by anti-CD4 FITC mAb and anti-FITC multisort beads. CD62L^{hi} T cells were positively selected using anti-CD62L microbeads. Antigen-presenting cells (APCs) were prepared by depletion of CD90$^+$ cells from the CD4-depleted cell fraction and irradiated (30 Gy) before culture. For sorting, an AutoMACS (Miltenyi Biotech, Bergisch Gladbach, Germany), or LS columns on MidiMACS separators (Miltenyi Biotec), were used according to the manufacturer's instructions.

To generate OVA TCR^{tg} Th1 cells, naive T cells were isolated from OT-II mice and activated by APCs in a ratio of 1:4 and 0.5 μM $OVA_{323-339}$ peptide (Biochemistry Department, Charité Universitätsmedizin, Berlin, Germany) in the presence of Th1 polarizing cytokines (as specified below). Non-transgenic naive T cells were activated on plate-bound anti-CD3/anti-CD28 mAbs. T cell cultures were split 1:2 after 3 days. Cytokines and antibodies were added to the media at the beginning and after splitting, unless otherwise stated. For Th1 polarization, IL-12 (5 ng/ml), IFNγ (20 ng/ml), and anti-IL-4 mAb (5 μg/ml) was added; for Th2 polarization, IL-4 (30 ng/ml), anti-IL-12, and anti-IFNγ mAb (5 μg/ml) was added. Th0 cultures contained anti-IL-12, anti-IFNγ, and anti-IL-4 mAb. IL-2 (10 ng/ml) was added to all cultures. For the STAT4 and T-bet chromatin immunoprecipitation, the cultures were supplemented only with IL-2 during the expansion phase.

2.4. Cytometric analysis and FACS sorting

Cells were stained with the respective antibodies in the dark at 4 °C for 15 min. P-lig staining using a P-selectin-human IgG chimeric protein and intracellular staining were performed as previously described (Kretschmer et al., 2004). For cytometric analysis, a LSRII or LSR Fortessa cytometer (BD Biosciences, Franklin Lakes, NJ) with FlowJo Software (Treestar Inc., Ashland, USA) was used. For FACS sorting, a FACSAriaII (BD Biosciensces) was used.