Immunology Letters 160 (2014) 133–138

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

ZSWIM1: A novel biomarker in T helper cell differentiation

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ARTICLE INFO

Article history:
Received 16 December 2013
Received in revised form 23 January 2014
Accepted 28 January 2014
Available online 4 February 2014

Keywords:
SWIM domain
T helper cells
Th17 cells
IL-17A
Th1 cells
Inflammation

ABSTRACT

The effector memory CD4+ Th17 cells play critical roles in bacterial immunity and pathological inflammation in autoimmune conditions. *ZSWIM1* is a gene encoding a protein of unknown function in leukocytes—but containing a zinc finger SWIM motif. In peripheral blood mononuclear cells, the expression of *ZSWIM1* is highest in lymphocytes, and in particular shows greatest abundance in naive CD4+ T cells. Upon polarisation of naïve CD4+ T cells, *ZSWIM1* expression is retained in Th17 cells but is selectively down regulated in Th1 cells. Similarly in *in vitro* expanded effector memory CD4+ T cells, ZSWIM1 was more abundant in Th17 cells compared to Th1 or Th17 polyfunctional (Th17pf) cells, which produce IL-17A and IFNγ. Although stimulation of cytokine production by PMA and ionomycin reduced *ZSWIM1* expression, the relative differences in abundance between the cell types were maintained. The activation sensitive nature of *ZSWIM1* expression suggests that it may play a novel role in the development or function of T helper cells.

1. Introduction

Understanding the roles of different leukocyte lineages and in particular functional subsets of T cells in normal and pathological immunity has been the cornerstone of modern immunology. The study of functional cell subsets has depended heavily on the identification of cell surface or intracellular molecules that have a restricted or ideally, exclusive expression on particular cell types. The investigation of functional characteristics including surface markers, cytokine production and nuclear factors, has led to the identification of a number of T helper cells including: Th1 and Th2 cells which are the major players in response against bacterial and viral foreign pathogens [1–3], Th17 cells which mediate inflammation [4–6], as well as T regulatory cells [7,8], T follicular helper cells [9], and suggested classifications for Th22 [10,11] and Th9 [12,13] cells.

While playing an essential role in normal immunity, Th17 cells have also been implicated in the development of human autoimmune diseases [14–17]. The Th17 cell is well defined as producing the cytokine IL-17A, but it is also capable of secreting many other cytokines such as IL-17F, CCL20, GM-CSF, and IL-6 in an inflammatory response [18,19]. Surface markers that are commonly used to identify Th17 cells include CCR6, CCR4 and CD161 [20–22]. In addition to surface markers, intracellular molecules such as cytoplasmic IL-17A and the transcription factor RORγt (encoded by *RORC*) that promotes *IL-17A* transcription are particularly useful markers of Th17 cells [22–24].

Considerable efforts are being made to identify novel molecules associated with the development and function of leukocyte subsets. Various systems biology approaches [21,25] have identified many genes and proteins of little or no known functional characterisation including the ZSWIM family of proteins.

The zinc finger, SWIM-type containing 1 gene, or *ZSWIM1* is located on chromosome 20 of the human genome. It has a transcript length of 2787 bp. There is one identified synonymous single-nucleotide polymorphism (SNP) at position 905, annotated in the ENTREZ SNP data base [26]. The gene encodes a protein of 485 amino acids which is predicted to only contain one functional domain. The SWIM domain derives its name from the SWI2/SNF2 family of ATPases and MuDr plant transposases, as the newly identified domain was found to have the closest alignments to these

Abbreviations: PBMCs, peripheral blood mononuclear cells; ICS, intracellular staining; PMA, phorbol 12-myristate 13-acetate; IFNγ, interferon gamma; qPCR, quantitative Real time polymerase chain reaction; Th, T helper; Th17pf, Th17 polyfunctional.

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http://dx.doi.org/10.1016/j.imlet.2014.01.016

two protein families in both prokaryotes and eukaryotes [27]. The SWIM domain is defined by the motif sequence $CxCx_nCxH$, where $n = 5–39$ amino acids [27]. The cysteine and histidine residues imply this domain is likely to adopt a zinc finger-like structure [28,29].

While the specific function of the ZSWIM1 is unknown, the presence of a zinc finger-like structure taken together with the observation that other proteins containing SWIM domains interact with other macromolecules suggests that ZSWIM1 may mediate its function by protein-protein interactions or DNA binding capabilities [30,31].

In this study we define the expression of human *ZSWIM1* in leukocytes and demonstrate the differential modulation of *ZSWIM1* expression during Th polarisation. We analysed the expression in *in vitro* expanded Th17 cells, Th1 cells and Th17 polyfunctional cells (co-producer of IL-17A and IFNγ).

2. Materials and methods

2.1. T helper cell isolation and culture

Memory CD4+ Th17 cells (capable of exclusively expressing IL-17A), Th1 cells (solely expressing IFNγ) and Th17 polyfunctional cells (capable of co expressing IL-17A and IFNγ) were isolated and expanded in culture as described previously [32], from healthy donor peripheral blood, obtained from the Australian Red Cross Blood Services. Expression of cytokines was determined by intracellular staining after PMA, ionomycin and brefaldin A incubation. Intracellular flow cytometry analysis was conducted using IL-17A PE (eBio64CAP17) (eBiosciences) and IFNγ AlexaFluor 647 (B27) (BD Biosciences) utilising the BD Cytofix/Cytoperm™ Fixation/Permeabilisation Solution Kit with BD GolgiPlug™ (BD Biosciences) following the manufacturer's specifications. Cells were analysed on the BD FACSCANTOII. Flow cytometry data was analysed using FlowJo (TreeStar).

2.2. Naïve CD4+ T cell culture

Naïve CD4+ T cells and memory CD4+ T cells were isolated by cell sorting using the surface markers CD4 FITC (RPA-T4), CD45RO Pe-Cy7 (UCHL-1), CD45RA APC (HI100), CD25 PE (M-A251)—all antibodies were obtained from BD Biosciences. Sorted cells were distributed into 96 well U bottomed plates at a concentration of $1–2 \times 10^5$ cells per well. Cells were cultured with αCD3/αCD28 TCR activator beads (Invitrogen Dynal) at a ratio of 1 bead: 3 cells. Cytokine cocktail mixes consisting of IL-23 (25 ng/ml), IL-1β (20 ng/ml), IL-6 (50 ng/ml), TGFβ (5 ng/ml), IL-12 (10 ng/ml), IL-2 (20 U/ml) (all from R&D Systems) were added to various cell cultures. Additionally, the following neutralising antibodies were used: αIFNγ (10 μg/ml) and αIL-4 (10 μg/ml) (BD Biosciences). The Th17 polarising condition contained: IL-23, IL-1β, IL-6, TGFβ, αIFNγ and αIL-4. The Th1 polarising condition contained: IL-12 and αIL-4. Cells were cultured for 7 days with replenishment of media and cytokines on day 4. Cells were then analysed by flow cytometry and RNA was extracted for qPCR analysis.

2.3. Isolation of peripheral blood mononuclear cell subsets

Additional leukocyte cell subsets from PBMCs were isolated by magnetic or fluorescence activated cell sorting. CD14+ Microbeads MACS kit (Miltenyi Biotech) and CD19+ PE antibody combined with anti-PE Microbeads MACS kit were used to isolate monocytes and B cells, respectively, by positive selection enrichment using magnetic separation. The antibodies CD8+ V450 (RPA-T8) and CD16+ FITC (3G8) were utilised for separation of the CD8+ T cells and CD16+ non-classical monocyte subsets by FACS.

2.4. Quantitative polymerase chain reaction analysis

Quantitative polymerase chain reaction (qPCR) was used to analyse the differential gene expression levels of genes of interest. RNA isolation was conducted with the RNeasy Mini Plus Kit (QIAGEN). Analysis of RNA concentration and quality was performed by spectrophotometry using the NanoDrop 2000 (Thermo Scientific). All RNA samples were stored at −70 °C. cDNA synthesis from the RNA of interest was performed using the AffinityScript QPCR cDNA Synthesis Kit (Agilent), random primers were used in the reaction. cDNA was then used immediately after synthesis in qPCR assays or stored at −20 °C for later use.

Primers for the qPCR reaction were designed using Primer 3 (http://primer3.sourceforge.net/) with the criteria of optimal melting temperature (T_M) of 60–62 °C, and an optimal primer length of 20 base pairs. Primer sets were evaluated by BLAST searches (NCBI) to verify their specificity and lack of secondary structures. The following primers were obtained from Sigma: *RORC:* 3′ GTTCCCACATCTCCCACATG, 5′ CCTGACAGAGATAGAGCACC [33]; *ZSWIM1:* 3′ AGACTCTCTCCCATGCTGGA, 5′ ACCGGACCTATAACCCAAGG; *GAPDH:* 3′ TGGTGGTCCAGGGGTCTTAC, 5′ ACCCACTCCTCCACCTTTGA. qPCR assays were conducted using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent), according to manufacturer's protocols. Data was analysed using MxPro (Stratagene), then exported for further calculations and graphing in Excel (Microsoft) and Prism (GraphPad Software). The relative difference in expression for a particular sample was calculated on the basis of the CT value obtained for that sample compared to the CT value of the comparator control, having an arbitrary expression value of 1. In the analysis between samples, the mean fold difference was calculated across multiple experiments with cells obtained from different donors. The p value for the mean fold difference values between compared samples was calculated with an unpaired two tail t-test, with p value <0.05 indicating statistical significance.

3. Results and discussion

3.1. ZSWIM1 expression is higher in Th17 cells

We had previously established conditions for the *in vitro* expansion of human Th17, Th1 and Th17 polyfunctional (Th17pf) cells [32]. In that study, the Th17 and Th1 cells were defined by their commitment to PMA and ionomycin induced expression of intracellular IL-17A and IFNγ, respectively, and in the case of Th17 polyfunctional cells by their capacity to synthesise high levels of both cytokines. A genome wide comparison of gene expression in these *in vitro* expanded Th17 and Th1 cells (Ko et al. in preparation) suggested that a gene encoding ZSWIM1, a protein of unknown function, was expressed at significantly greater levels in Th17 cells.

This relative difference in expression between Th17 and Th1 cells was confirmed by qPCR analysis of *ZSWIM1* mRNA in Th17, Th1 and Th17pf cells with and without stimulation by PMA and ionomycin (Fig. 1). The differences in *ZWIM1* mRNA levels were determined by comparison of the levels in Th17 or Th17pf relative to the levels in Th1 cells which were arbitrarily assigned an expression value of 1. Thus the expression of *ZSWIM1* was 4.2 fold higher in unstimulated Th17 cells relative to Th1 cells but the *ZSWIM1* mRNA expression levels detected in Th17 polyfunctional cells and Th1 cells were not significantly different.

Since the induction of cytokine synthesis in these expanded cells is dependent on their stimulation, we investigated the effect of cytokine inducing stimulus on the relative levels of *ZSWIM1* mRNA. Stimulation with PMA and ionomycin down regulated *ZSWIM1* expression in all three cell subsets, thus *ZSWIM1* expression was