Molecular Immunology 54 (2013) 199–207

Contents lists available at SciVerse ScienceDirect

Molecular Immunology

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

Podoplanin is an inflammatory protein upregulated in Th17 cells in SKG arthritic joints

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ARTICLE INFO

Article history:
Received 10 October 2012
Received in revised form
21 November 2012
Accepted 27 November 2012
Available online 31 December 2012

Keywords:
Helper T cell
Rheumatoid arthritis
Cell surface molecules

ABSTRACT

Interleukin 17-producing helper T (Th17) cells play pathogenic roles in chronic inflammatory and autoimmune diseases, including arthritis, colitis and multiple sclerosis. Th17 cells selectively express the transcription factor RORγt, as well as the cytokine receptors IL-23R and CCR6. Identification of novel Th17 cell-specific molecules may have potential value as diagnostic markers in the above-mentioned inflammatory diseases. To that aim, we carried out a comparative microarray analysis on *in vitro* differentiated Th1, Th2, Treg and Th17 cells from naïve $CD4^+$ cells of BALB/c mice. Among a total of one hundred and twenty Th17 cell-specific molecules, twenty-nine were novel cell-surface molecules. Then we revealed that thirteen of them were up-regulated *in vivo* in inflamed tissues from experimental autoimmune diseases, including spontaneous SKG arthritis, inflammatory bowel disease (IBD) and experimental autoimmune encephalomyelitis (EAE). Next, we analyzed the expression of four membranous molecules, and revealed that podoplanin was expressed highly in the *in vitro* differentiated Th17 cells. Moreover, at the inflamed synovium of the arthritic SKG mice, most of the accumulating Th17 cells were podoplanin-positive. These results indicate that podoplanin would be a useful Th17 cell marker for diagnosing pathological conditions of autoimmune diseases, including rheumatoid arthritis.

1. Introduction

Helper T (Th) cells play critical roles in acquired immune responses. Naïve Th cells differentiate into effector Th cells when stimulated by antigen-MHC complexes on antigen presenting cells, such as dendritic cells, macrophages and B cells. Depending on the type of environmental cytokines, activated Th cells differentiate into functionally distinct subsets, characterized by different cytokine and transcription factor profiles (Bettelli et al., 2008; Dong and Flavell, 2001; Glimcher and Murphy, 2000; Miossec et al., 2009; Mosmann and Coffman, 1989). Along with classical Th1 and Th2 cells, another subset of Th cells, denominated as Th17 cells, has attracted much attention because of their connection with both infection and autoimmune diseases (Korn et al., 2009). The main physiological function of IL-17, the signature cytokine of Th17 cells, is protection from infectious diseases. Indeed, IL-17-deficient mice are highly susceptible to infection by *Citrobacter rodentium* (Ishigame et al., 2009) and *Candida albicans* (Huang et al., 2004). Similarly, mice that lack the expression of IL-17RA have severe attenuation in host defense against *Klebsiella* (Ye et al., 2001) and *Candida* (Saijo et al., 2010). Th17 cells protect against bacterial and fungal infection through several mechanisms: (1) expression of CCR6 and IL-23R on the cell surface and migration to intestinal lymphoid tissues and inflammatory sites, (2) production of inflammatory cytokines and chemokines, such as IL-17A, IL-17F, IL-22 and CCL20. The induction of Th17 differentiation is induced by IL-6 and TGF-β through upregulation of RORγ, and the Th17 phenotype is maintained and stabilized by IL-23 released from antigen presenting cells, as Th17 cells do not produce IL-6, TGF-β or IL-23 necessary for their own differentiation. (Acosta-Rodriguez et al., 2007; Aggarwal et al., 2003; Ivanov et al., 2006; Wilson et al., 2007; Zhou et al., 2007).

The roles of Th17 cells have become clear over the past decade, based on results from experimental animal models of inflammatory diseases, as well as from clinical studies in human. The pathogenesis of rheumatoid arthritis (RA), like that of several other chronic inflammatory diseases such as Crohn's disease, psoriasis and multiple sclerosis, is linked to the deleterious roles of Th17

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0161-5890/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.molimm.2012.11.013

cells (Annunziato et al., 2009; Miossec et al., 2009; van den Berg and Miossec, 2009). IL-17-deficient mice show suppression of development of collagen-induced arthritis (Nakae et al., 2003). Moreover, the SKG strain of mice, a mutant on BALB/c background, spontaneously develops Th17-mediated autoimmune arthritis (Hirota et al., 2007a; Sakaguchi et al., 2003). It was shown in this model that CCR6 and CCL20 expressed by Th17 cells and inflamed synovial tissues, respectively, are required for the migration of Th17 cells, leading to self-destructive immune reactions in the joints (Hirota et al., 2007b). Furthermore, adoptive transfer of splenic Th cells of SKG mice into BALB/c mice induces arthritis, whereas that of splenic Th cells of IL-17-deficient SKG mice does not (Hirota et al., 2007a).

In arthritic mice, most cells in the joint tissues express IL-17R, and IL-17 activates synovial cells and inflammatory migrating cells. Activated Th17 cells induce destruction of the extracellular matrix and bone by activation of osteoclasts. The receptor activator of NF-κB ligand (RANKL) on Th17 cells activates the receptor activator of NF-κB (RANK) on osteoclasts, leading to enhanced bone destruction (Miossec et al., 2009).

Moreover, IL-17 levels in the serum and synovial fluid significantly correlate with disease activities of RA patients (Metawi et al., 2011). Indeed, results from a phase I clinical trial show significant beneficial effects of a humanized anti-IL-17 mAb in patients with RA (Genovese et al., 2010; Leonardi et al., 2012; Miossec et al., 2009; Waite and Skokos, 2012). This is underscored by two very recent reports on phase II clinical trials demonstrating the above-mentioned anti-IL-17 mAb, as well as an mAb specific for the IL-17R in the treatment of psoriasis (Genovese et al., 2010; Leonardi et al., 2012; Miossec et al., 2009; Papp et al., 2012; Waite and Skokos, 2012). Taken together, these data confirm that Th17 cells play a critical role in the pathogenesis of RA and other chronic inflammatory disorders. This leads to the assumption that the identification of novel Th17 cell-specific molecules may be of diagnostic or therapeutic value in the treatment of such diseases (Genovese et al., 2010; Leonardi et al., 2012; Miossec et al., 2009; Waite and Skokos, 2012).

In the present study, we have improved the experimental conditions of *in vitro* differentiation of mouse naïve Th cells, obtained highly differentiated Th populations, and subsequently performed comparative microarray analysis. Thus, we identified podoplanin as a cell surface molecule highly expressed by Th17 cells. We confirmed its expression in the inflamed tissues of experimental animal models of arthritis, autoimmune encephalomyelitis (EAE) and inflammatory bowel disease (IBD). Our results indicate that podoplanin might be a pathogenic Th17 cell marker and useful for diagnosis of autoimmune diseases.

2. Materials and methods

2.1. Reagents

Recombinant mouse IL-2, IL-12, IL-4, IL-1β, IL-23 and TNF-α were purchased from R&D Systems. Recombinant mouse IL-6 and human TGF-β, and Abs to IFN-γ FITC (XMG1.2), CD62L FITC (MEL-14), CD25 PE (PC61), IL-17A PE (TC11-18H10), CD44 APC (IM7), IL-4 APC (11B11), and CD4 PEcy7 (RM4-5) were purchased from BD. Abs to Foxp3 PE (150D), CCR6 Alexa Fluor® 647 (29-2L17), CD8a (53–6.7), B220 (RA3-6B2), CD11b (M1/70), Gr1 (RB6-8C5), Ter119 (TER-119) were purchased from Biolegend. Abs to FcγRIIb PE (190909) and podoplanin (R&D Systems), Ab to HVEM PE (LH1) (eBioscience) and Ab to cannabinoid receptor 2 (CNR2) (Abcam) were purchased. Alexa Fluor® 488-conjugated anti-goat IgG and anti-rabbit IgG were purchased from Invitrogen. FITC- and PE-conjugated isotype controls were purchased from BD. Other conjugated isotype controls were purchased from BioLegend.

2.2. Mice

Female SKG/Jcl mice (CLEA Japan, Inc.), SJL/J mice, BALB/cAnN CrlCrlj and CB17/Icr-Prkdc<scid>/CrlCrlj mice were purchased from Charles River Laboratories Japan, Inc. The mice were kept under specific pathogen-free conditions in the Oriental BioService, Inc., Kobe BioMedical Laboratory, and treated in a humane manner in compliance with the guidelines of the Animal Experimental Committee of Sysmex Corporation and Oriental BioService, Inc.

2.3. Induction of arthritis, EAE and IBD

Arthritis was induced by injecting SKG mice i.p. with 10 mg of curdlan from *Alcaligenes faecalis* (Sigma–Aldrich) emulsified in PBS, and with 10 mg of curdlan from *A. faecalis* 4 weeks later. The severity of arthritis was measured as described previously (Sakaguchi et al., 2003). Mice showing a score of more than three were included in the analysis.

Acute relapsing EAE was induced by injecting SJL mice s.c. with 200 μl of emulsion containing 200 μg of PLP (139–151) peptide and 4 mg of mycobacterium tuberculosis extract H37Ra (Difco) in incomplete Freund adjuvant (Difco). In addition, 200 μg of Pertussis toxin (List Biological Laboratories, Inc.) was administered i.v. on day 0. Clinical signs of EAE were assessed according to the following scores, as described: 0, no signs of disease; 1, loss of tone in the tail; 2, hind limb paresis; 3, hind limb paralysis; 4, tetraplegia; 5, moribund. Mice with a score of more than two were analyzed (Stromnes and Goverman, 2006).

IBD was induced by injecting SCID mice i.p. with 4×10^5 naïve T cells, derived from BALB/c mice. The severity of colitis was estimated by measurement of the body weight. Mice with a loss of more than twenty percent were included in the study (Read and Powrie, 2001).

2.4. Isolation and differentiation of naïve T cells

Splenic T cells were enriched from 8- to 10-week-old BALB/c mice by negative depletion using a cocktail of 0.5 μg/ml of anti-CD8a, anti-B220, anti-CD11b, anti-Gr1 and anti-Ter119 mAbs, followed by incubation with goat anti–rat IgG–coated beads (BioMAG®, Polysciences, Inc.) and enrichment on a magnetic particle separator. Naïve Th cells ($CD4^+CD25^-CD62L^{high}CD44^{low}$) were further purified using a FACS Aria™ (BD) with a purity of >99%. After activation on plate-bound anti-CD3 and 10 μg/ml soluble anti-CD28 for 3 days, Th cells were cultured for 4 more days in RPMI-1640, supplemented with 10% FBS, 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen) and 50 μM 2-ME (Sigma–Aldrich). Then, the Th cells were restimulated with plate-bound anti-CD3 and 10 μg/ml soluble anti-CD28 for 3 days and cultured for 4 days before analysis. The following cytokines and neutralizing antibodies were added for *in vitro* Th17 lineage differentiation: 20 ng/ml rIL-6, 5 ng/ml rTGF-β, 10 ng/ml rIL-2, and 10 μg/ml anti-IFN-γ and anti-IL-4 mAbs (for the experimental conditions previously reported) (Bettelli et al., 2006; Mangan et al., 2006); and 10 ng/ml rIL-1β, rIL-23, rTNF-α, 20 ng/ml rIL-6, 5 ng/ml rTGF-β, as well as the combination of anti-IFN-γ, anti-IL-4 and anti-IL-2 mAbs at a concentration of 10 μg/ml (for the condition established in the present study). For Th1 or Th2 lineage differentiation, naïve T cells were stimulated and cultured in the presence of 10 ng/ml rIL-12 and 10 μg/ml anti-IL-4 mAb or 10 ng/ml rIL-4 and 10 μg/ml anti-IFN-γ mAb, respectively. For Treg cell differentiation, 5 ng/ml rTGF-β and the combination of anti-IFN-γ, anti-IL-4, anti-IL-6 mAbs (each at 10 μg/ml) were added. Th1, Th2 and Treg of the above cultures contained 10 ng/ml rIL-2.