Molecular Immunology 58 (2014) 38–49

Contents lists available at ScienceDirect

Molecular Immunology

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

Active demethylation of the IL-2 Promoter in CD4+ T cells is mediated by an inducible DNA glycosylase, Myh

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ARTICLE INFO

Article history:
Received 15 August 2013
Received in revised form 23 October 2013
Accepted 29 October 2013
Available online 26 November 2013

Keywords:
Demethylation
IL-2
DNA glycosylase
Myh
CD4+ T cells

ABSTRACT

Epigenetic control of tissue-specific gene expression is often achieved by active demethylation of promoter regions; however, the nature of all the enzymes mediating this remodeling process is not fully clear. Here we describe a 5-methylcytosine glycosylase activity for the murine DNA base excision repair enzyme Myh and show that it is critically involved in remodeling the IL-2 Promoter for transcription. The enzyme is not expressed in naïve CD4+ T cells, but can be transiently induced following T cell activation. T cells deficient in Myh had blunted demethylation of the promoter and impaired IL-2 secretion but not IFN-γ. An in vitro assay for the glycosylase activity revealed the enzyme to be sequence specific for certain CpG sites in the IL-2 Promoter. These results suggest that DNA demethylation is being selectively used to orchestrate a part of the naïve CD4+ T cell differentiation program.

1. Introduction

The expression of the *il2* gene in T cells is very precisely induced in response to stimulation of the T cell receptor (TCR) with antigen/MHC complexes and co-stimulation by antigen-presenting cells. The promoter region of *il2* is methylated at CpG sites in naïve T cells and is rapidly demethylated upon T cell activation, facilitating *il2* gene transcription (Bruniquel and Schwartz, 2003; Thomas et al., 2005; Murayama et al., 2006). This genetic remodeling occurs long before the initiation of cell division in the stimulated T cell, making this system a useful tool to dissect the mechanism underlying this active DNA demethylation in mammalian cells. Recent progress in identifying DNA demethylases in plants and vertebrates has implicated DNA glycosylases in a base excision repair-mediated mechanism for demethylation (with or without prior deamination) (Yoon et al., 2003; Hendrich et al., 1999; Zhu et al., 2000; Rai et al., 2008; Kim et al., 2009; Jiricny and Menigatti, 2008; Gong et al., 2002; Gehring et al., 2006; Agius et al., 2006; Morales-Ruiz et al., 2006; Zhu, 2009; Hajkova et al., 2010). In addition, the Tet family of DNA dioxygenase enzymes has been shown to initiate demethylation through oxidation of the 5-methylcytosine (Tahiliani et al., 2009; Ito et al., 2010; He et al., 2011). We demonstrate here that the murine DNA base excision repair glycosylase Myh can also act as a DNA demethylase. The protein is rapidly induced in naïve CD4+ T cells upon stimulation, and functions to actively demethylate specific CpG sites in the IL-2 Promoter, which facilitates the initiation of IL-2 production.

2. Materials and methods

2.1. Antibodies and chemicals

The affinity purified goat polyclonal IgG antibody raised against a peptide located in the C-terminus of mouse Myh was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA [MUTYH(M-15), catalog #sc-30633]. The affinity purified goat polyclonal IgG antibody raised against a C-terminal peptide of human actin, which cross-reacts on mouse actin, was also purchased from Santa Cruz Biotechnology, Inc. [Actin (C-11) catalog #sc-1615]. Both of these antibodies were detected in Western blots with a secondary donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc., catalog #2020). The anti-CD3 monoclonal antibody 145-2C11 was purchased from BD Pharmingen, Franklin Lakes, NJ (catalog #553057). The anti-CD28 ascites fluid we used was prepared from a monoclonal antibody hybridoma (clone 37.51) originally generated in the laboratory of Dr. James Allison (Gross et al., 1990). The three flow cytometry staining monoclonal antibodies: anti-CD4-APC (clone RM4-5) anti-CD62L-FITC (clone MEL-14) and anti-CD44-PE (clone IM7) were purchased from BD Pharmingen. The chemicals Phorbol Myristate Acetate (PMA), ionomycin, 4′-6-diamidino-2-phenylindole (DAPI), actinomycin D (Act-D), and cycloheximide (CHX) were all purchased from Sigma-Aldrich,

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http://dx.doi.org/10.1016/j.molimm.2013.10.016

St Louis, MO, catalog #s P-8139, I-0634, 28718-90-3, A1410, and C4859, respectively. The SssI ^{5}CpG methyltransferase and S-adenosylmethionine (SAM) were obtained from New England Biolabs, Inc., Ipswich, MA (catalog #MO226L and B9003S, respectively).

2.2. Mouse strains

The B10.A Rag2$^{-/-}$ TCR 5C.C7 transgenic mouse was derived as previously described (Miller et al., 1999). The BALB/c Rag2$^{-/-}$ TCR DO11.10 transgenic strain was derived from the original gene-targeted line (Murphy et al., 1990) backcrossed 12 times onto a BALB/c background and then once to the BALB/cTac Rag2$^{-/-}$ strain. The B6/129 mixed background Myh$^{-/-}$ (Xie et al., 2004) and Ogg1$^{-/-}$ (Klungland et al., 1999) gene-targeted mice were the kind gift of Drs Jeffrey H. Miller and Robert Schiestl at UCLA. All mice were bred at the NIAID/Taconic contract in Germantown, NY, and housed in our AAALAC-approved, specific pathogen-free colony under animal study protocols approved by the NIAID Animal Care and Use Committee.

2.3. Preparation and stimulation of CD4^{+} T cells

Naïve CD4^{+} T cells were isolated from lymph nodes of BALB/c Rag2$^{-/-}$ TCR DO11.10 or B10.A Rag2$^{-/-}$ TCR 5C.C7 transgenic mice. Pooled CD4^{+} T cells from cervical, axillary, inguinal and mesenteric lymph nodes were used in these experiments. The purity of CD4^{+} T cells was greater than 90%. For the B6/129 Myh$^{-/-}$ and Ogg1$^{-/-}$ experiments CD4^{+} CD62L^{hi} CD44^{lo} T cells were sorted by flow cytometry from a pool of suspended spleen and lymph node cells. The CD4 purity was 95–99%. In some experiments (e.g. those with B6/129 Myh$^{-/-}$ mice) the total CD4^{+} T cell pool was isolated by magnetic bead negative selection using a CD4^{+} T Cell Isolation Kit II (catalog #130-095-248) Miltenyi Biotec, Auburn, CA, or a Dynal Mouse CD4 negative isolation kit (catalog #114.15D) Invitrogen Dynal AS, Oslo, Norway. The purity in these cases was around 90%. In one set of experiments NK cells (DX5^{+}) were isolated from the same spleen cells using a Miltenyi Biotec NK cell isolation kit (catalog #130-090-864). Each CD4^{+} T cell population was stimulated with either plate bound anti-CD3 antibody (1 or 3 μg/ml coating) plus soluble anti-CD28 antibody (1/5000 dilution of ascites) or a combination of PMA (10 or 40 μg/ml), ionomycin (0.5 μg/ml), and anti-CD28 ascites. Ten thousand cells in a 96 well or 2.7 million cells in a 6 well flat-bottom plastic plate (Costar #3596 or #3506, Corning, NY) were cultured in 0.2 or 3 ml, respectively, of E/R medium (50% RPMI 1640/50% Eagle's Hank's Amino Acids (EHAA) from Invitrogen, Carlsbad CA) supplemented with 10% Fetal Calf Serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol. At 24, 48, or 72 h the 96 well culture supernatants were harvested and IL-2 and IFN-γ cytokine levels measured by an enzyme-linked immunosorbent assay (ELISA kits from R&D Systems, Minneapolis, MN). At 16 h the cells were harvested from the 6 well cultures and analyzed by flow cytometry for cytokine secretion in an IL2-PE capture assay using a bifunctional antibody specific for CD45 and IL-2 (Miltenyi Biotec) (Sojka et al., 2004)

2.4. IL-2-GFP reporter constructs

The wild type (WT) and CpG-mutated (Thomas et al., 2005; Murayama et al., 2006; Yoon et al., 2003; Hendrich et al., 1999; Zhu et al., 2000; Rai et al., 2008; Kim et al., 2009) IL2-GFP reporter constructs were made with an 8.4-kb IL-2 Promoter/enhancer (Yui et al., 2004) driving expression of enhanced green fluorescent protein (GFP). CpG mutations of sites 2–8 in the *il2* proximal promoter were made with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The 7 mutations were confirmed by DNA sequencing. These constructs were made by Dr. Denis Bruniquel while he was a member of LCMI/NIH.

2.5. Plasmid and oligomer methylation and demethylation in vitro

Fully methylated plasmids and oligomers were made in vitro by mixing the unmethylated DNA with the *Sss*I methyltransferase and its co-substrate S-adenosylmethionine (SAM) (New England Biolabs, Inc.). In some experiments a tritium-labeled (5 μCi of 15 Ci/mmol) version of SAM was used (GE Healthcare, Piscataway, NJ or Perkin Elmer, Waltham, MA). Twenty microgram of DNA were incubated for 16 h at 37 °C with 16 U of *Sss*I and a 3.2 mM final concentration of SAM. After completion of the reaction, the methylated plasmids were recovered with a mini-prep DNA purification kit (catalog #27106 from Qiagen, Valencia CA).

To study demethylation of the IL-2-GFP plasmids, 10 μg of DNA were incubated with 2 μg of some of the known DNA glycosylases: Myh (Yang et al., 2001), Tdg (Neddermann et al., 1996), Fpg1 (Dou et al., 2003), Fpg2 (Dou et al., 2003), Ogg1 (Radicella et al., 1997), or the ^{m5}CpG binding protein Mecp2 (Bhattacharya et al., 1999). Each of these molecules was expressed as a His-tagged recombinant protein in E. coli using the pET system (Catalog #71329, EMD chemicals, Gibbstown, NJ) and purified on a nickel column (BugBuster His•Bind Purification Kit, Catalog #70793, EMD Chemicals) For a few experiments, the Myh enzyme was further purified to 90% purity by Fast Protein Liquid Chromatography (FPLC). After incubation at 37 °C for up to 2 h, the reaction mixture was stopped by heating to 75 °C for 10 min, run on a 1% agarose gel and stained with ethidium bromide to monitor for plasmid migration and digestion.

To study demethylation using the defined oligomers, the PCR fragments were labeled with ^{32}P-ATP using a T4 DNA kinase (catalog #M0201S, New England Biolabs, Inc.) for 30 min at 37 °C. The labeled probes were isolated by passing through a ProbeQuant G-50 Micro column (catalog #27-5335-01, GE Healthcare) and then incubated with the recombinant Myh enzyme at 37 °C for various times. The reaction was stopped by heating the sample to 100 °C for 10 min in 0.2% NaOH, in order to denature the DNA into single strands, and then chilled on ice to preserve this form. Finally, the mixture of digested and undigested ssDNA was separated by electrophoresis in urea on a 15% polyacrylamide DNA sequencing gel and the radioactive bands detected with Kodak Biomax MS film. The synthetic oligos were assayed in a similar manner, although sometimes in a buffer without NaOH.

For the experiments with ^{3}H-methyl-SAM, the reaction was stopped after various times up to 2 h by the addition of EDTA (5 μl of a 0.5 M stock solution) and the mixture separated on a Qiagen mini-prep column. The flow-through liquid containing any released tritium, and the bound DNA that was subsequently eluted with water, were both counted in a beta liquid scintillation counter (Perkin–Elmer, Waltham, MA). Total (bound plus flow-through) cpm recovered was similar at all time points in the kinetic analysis. For experiments examining the nature of the released tritium-labeled material, the flow-through liquid was concentrated in a Spin-Vac Vacfuge plus (Eppendorf, Hamburg), disolved in water and spotted onto a silica gel Thin Layer Chromatography plate (Silicagel 60 F254, 20 cm × 20 cm, Merck, Rahway, NJ), along with 10 μl of a concentrated solution of unlabeled 5-methylcytosine and thymine (Morales-Ruiz et al., 2006). Chromatography was performed in two dimensions using chloroform: methanol:water (4:2:1 vol) in the first dimension and ethylacetate:propanol:water (75:16:9 vol) in the second dimension, with air drying in between. The 5-methylcytosine and thymine spots were then identified under UV light, scraped off separately, and counted in a beta liquid scintillation counter.