HHS Public Access

Author manuscript

Mol Immunol. Author manuscript; available in PMC 2015 July 27.

Published in final edited form as:

Mol Immunol. 2012 September ; 52(2): 71–78. doi:10.1016/j.molimm.2012.04.011.

TCR stimulation upregulates MS4a4B expression through induction of AP-1 transcription factor during T cell activation

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Abstract

MS4a4B is a novel member of the membrane-spanning 4-domain family, subfamily A (MS4A) specifically expressed in mouse T cells. We have shown previously that expression of MS4a4B in T cells is upregulated upon T cell activation, suggesting that MS4a4B may play a functional role in regulation of T cell responses. However, little is known about the mechanisms that regulate MS4a4B gene expression. In this study, we explored the potential mechanism underlying TCR-stimulation-induced expression of MS4a4B by promoter analysis. We cloned 2495 bp of 5′-flanking region upstream of the MS4a4B start code and inserted the DNA fragment into pGL4.20 reporter plasmid. To analyze promoter activity of the cloned DNA fragment, we transiently transfected EL4 thymoma cells and the T32 cell line with reporter plasmids. Expression of reporter gene was determined by dual-luciferase assay. Potential activator- and repressor-binding sites were analyzed by serial length of 5′-deletion. We have identified at least two potential activator binding regions and two potential repressor binding regions. The activator binding sites have been localized to two fragments, which are a 442-base pair region (region A) positioned from −1176 to −735, and a 119-base pair region (region B) positioned −188 to −70 respectively. MatInspector analysis showed that region A contains the consensus binding motif of the AP-1 family of transcription factors. Machinery analysis showed that nuclear proteins extracted from anti-CD3/anti-CD28-activated primary T cells specifically bind to the AP-1 binding element. In contrast, blockade by AP-1 inhibitor in culture decreased MS4a4B expression in T cells. Our data demonstrate that TCR-stimulation induces transactivation of AP-1 transcription factor, which subsequently binds to the MS4a4B promoter and upregulates expression of MS4a4B in activated T cells.

Keywords

T cells; Gene promoter AP-1; MS4A family

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1. Introduction

In immune responses, T cell activation and proliferation are regulated at multiple levels by proliferative and anti-proliferative signals (Krammer et al., 2007; Mustelin and Tasken, 2003; Wells, 2003). It has been shown that resting T cells are activated and proliferate upon T cell receptor (TCR)-stimulation while the same stimulus also leads pre-activated T cells to undergo apoptosis (Dhein et al., 1995; Singer and Abbas, 1994). Proper regulation on T cell proliferation and activation therefore is necessary for maintaining appropriate function and physiological balance of the immune system.

In our previous studies, we found that MS4a4B, a tetra-span-transmembrane protein of the membrane-spanning 4-domain family, subfamily A (MS4A) (Ishibashi et al., 2001; Liang et al., 2001), is highly expressed in T cells and its expression is up-regulated during T cells activation (Xu et al., 2006). Moreover, over-expression of MS4a4B by viral vectors reduces proliferation of EL4 thymoma cells, while knockdown of MS4a4B promotes proliferation of primary T cells and T32 cells (a T cell line derived from primary Th1 cells) (Xu et al., 2010). These data indicate that MS4a4B plays an inhibitory role in proliferation of activated T cells, which may serve as a negatively regulatory mechanism for maintaining balance of the immune response system during TCR-induced T cell activation. However, it is not known how TCR stimulation leads to enhanced expression of MS4a4B in T cells. Based on our previous findings, we hypothesize that stimulatory signals through TCR may induce transactivation of certain transcription factors that bind to the MS4a4B promoter and enhance production of MS4a4B in activated T cells. To test this hypothesis, we cloned the MS4a4B promoter from C57BL/6 mice and analyzed activity of the promoter by reporter gene expression.

In this article, we analyzed the regulatory elements in the MS4a4B promoter. We demonstrated that TCR-triggered signals induce expression of transcription factor activator protein 1 (AP-1) during T cell activation. Transactivated AP-1 proteins bind to an activating motif of the MS4a4B promoter and enhance production of MS4a4B protein, which subsequently inhibits proliferation of activated T cells.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Female mice, 8–12 weeks old, were used in all studies. All experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Committee of Thomas Jefferson University.

2.2. Cells and culture conditions

The EL4 thymoma cell line was cultured in RPMI 1640 medium with 2 mM μ-glutamine, 10% fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 units/ml) and incubated in a stable environment of 5% CO_2 at 37 °C. T32 cells were cultured in the same medium in the presence of 20 U/ml mouse recombinant IL-2 (Roche Diagnostics, Indianapolis, IN).

2.3. In silico sequence analysis

Mouse genomic DNA sequences, 2495 bp upstream of the start code of the MS4a4B gene, were downloaded from the NCBI database [GenBank: NT 039687.7]. Possible transcription factor-binding sites were predicted on genomic DNA sequences using MatInspector software (Cartharius et al., 2005).

2.4. Preparation of the reporter constructs

Mouse genomic DNA was extracted from mouse tails using DNeasy™ Tissue kit following the manufacturer's instructions (Qiagen, Valencia, CA). The 2495 bp fragment in the 5′-flanking region of the mouse MS4a4B gene was amplified from mouse genomic DNA by PCR with specific primers to MS4a4B gene (sense primer P1 and anti-sense primer in Table 1). Other fragments with different lengths in the 5′-flanking region were amplified by PCR using the cloned 5′-flanking DNA as the template with primers shown in Table 1. All primers were synthesized by Integrated DNA Technologies (Coralville, Iowa). PCR was performed using AccuPrime™ Taq DNA polymerase (Invitrogen, San Diego, CA) as follows: 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 67 °C for 2.5 min, with a final extension at 67 °C for 7 min. The PCR products were gel-purified, digested with BamHI/XhoI, and subcloned into the pGL4.20 firefly luciferase vector (Promega, Madison, WI). The sequences of cloned promoter regions were confirmed by DNA sequencing. All plasmid DNA used for transfection was purified with Plasmid Midi Kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA).

2.5. Transfection and dual luciferase reporter assay

EL4 and T32 cells ($3–5 \times 10^6$) were transiently transfected with 10 μg of the reporter plasmid and 0.5 μg of the pRL-nul Renilla control vector (Promega, Madison, WI) that act as an internal control for the normalization of transfection efficiency. Electroporation was performed using the BioRad Gene Pulser Xcell™ system (BioRad, Hercules, CA), according to the manufacturer's protocols. Cells were harvested at 24 h posttransfection and lysed. Specific luciferase activity in the cell lysates was measured with a Veritas Microplate Luminometer (Promega, Madison, WI). All luciferase assays were performed at least three times, each in triplicate. The dual luciferase double reporter assay system and substrates were purchased from Promega.

2.6. Electrophoretic Mobility Shift Assays (EMSA)

Biotin-labeled and unlabeled oligonucleotides containing AP-1 binding motif (AP-1 sense-biotin: 5′-biotin-CCAAATGAGTAAGTTG- 3′; AP-1 sense: 5′-CCAAATGAGTAAGTTG-3′; AP-1 anti-sense: 5′-CAACTTACTCATTTGG-3′) were synthesized for EMSA. To generate double-stranded probes, complementary oligonucleotides were mixed together at a 1:1 molar ratio, heated to 95 °C and were then gradually cooled down to room temperature. Nuclear extracts were prepared from stimulated primary T cells according to methods described previously (Sadowski and Gilman, 1993). EMSAs were performed using the LightShift chemiluminescent EMSA kit (Thermo Scientific, Rockford, IL). Briefly, 0.1 pmol of each biotin end-labeled double-stranded probe

was incubated for 25 min at room temperature in 20 μl of EMSA binding buffer (10 mM Tris, 50 mM KCl, 1 mM dithiothreitol, pH 7.5) containing 2.5% Glycerol, 5 mM $MgCl_2$, 50 ng/μl poly(dI·dC), 0.05% Nonidet P-40, and 6 μg of nuclear proteins. For competition EMSA, 200-fold (20 pmol) excess unlabeled, double-stranded probe was added to the binding reaction. The DNA-nuclear protein complexes were resolved by electrophoresis in 7% non-denaturing polyacrylamide gel. Gels were then electroblotted onto Biodyne B nylon membranes (Thermo Scientific, Rockford, IL). The membranes were then cross-linked by a transluminator at 312 nm, and visualized using the chemiluminescent nucleic acid detection system (Thermo Scientific, Rockford, IL).

2.7. Chromatin Immunoprecipitation assay (ChIP)

ChIP assay was performed using Chromatin Immunoprecipitation (ChIP) Assay Kit (Upstate Cell Signaling Solutions, USA) according to the manufacturer's instructions. Briefly, purified $CD4^+$ T cells were activated with soluble anti-CD3 (1 μg/ml, BD Biosciences)/anti-CD28 (1 μg/ml, BD Biosciences) for 3 days. The activated $CD4^+$ T cells were fixed with formaldehyde, and sonicated to shear the chromatin and immunoprecipitated by using mouse anti-c-Jun (Cell Signaling, Danvers, MA) antibody-coupled Protein A-agarose-beads. Normal mouse IgG-coupled Protein A-agarose-beads were parallel performed as a control. The MS4a4B promoter fragment was amplified by PCR using specific primers to MS4a4B promoter DNA as follows: MS4a4B-ChIP-sense: 5′-AGGGAATGAATAGACAACCCAGGAG-3′, and MS4a4B-ChIP-anti-sense: 5′-GCCTACACTTCCTTTTCATGTTCCAG-3′.

2.8. Mutation of AP-1 binding site in the MS4a4B promoter

A construct with 8-bp mutation at AP-1 consensus binding sequence of the MS4a4B promoter in P3 luciferase reporter plasmid (Fig. 2B) was generated by PCR mutagenesis using the QuikChange site-directed mutagenesis kit according to manufacturer's instructions (Stratagene; Santa Clara, CA). A sense primer (5′-GGTTCTTTGTACCAATCCCCATTCATGTGCATTTCACTAG-3′) and its reverse complement as an anti-sense primer were synthesized to mutate AP-1 consensus sequence aatGAGTaagt into aatCCCCattc.

2.9. Real-time quantitative PCR

Total RNA was isolated from cultured cells with TriZol (Life Technologies, Gaithersburg, MD). For RT-PCR, 2 μg of total RNA was transcribed into cDNA using high capacity cDNA transcription kit (Applied Biosystems, Carlsbad, CA) according to manufacture's instructions. Quantitative real-time PCR was performed with specific primer sets using Applied Biosystems 7000 Real-time PCR system and SYBR Green detection chemistry. PCR reaction was performed (50 °C, 2 min; 95 °C, 10 min, followed by 40 cycles on 95 °C, 15 s; 60 °C, 1 min) and then melting curve reaction was performed to verify the specificity of amplification) using Power SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA). Transcript quantization was relative to HPRT standard. Error bars indicate SEM values calculated from −ΔΔ Ct values from triplicate PCR reactions according to Applied Biosystems protocols. Primers for MS4a4B were shown previously (Xu et al., 2006); primers for c-Jun: 5′-GTCTACGCCAACCTCAGCAACTTC-3′ and 5′-

CGGTCTGCGGCTCTTCCTTC-3′; primers for c-Fos: 5′-CTGTCCGT-CTCTAGTGCCAACTTTAT-3′ and 5′-GACTGGGTGGGGAGTCCGTAA-3′.

2.10. Western blotting

The primary T cells were stimulated on 24 well plate coated with anti-CD3 (5 μg/ml)/anti-CD28 antibody (2 μg/ml) for different periods and then were harvested. Cells were lysed by cell lysis buffer (Cell Signaling, Danvers, MA) supplemented with 1 mM phenylmethylsulfonyl fluoride and 1× proteinase inhibitor cocktails (Sigma, St. Louis, MO). Fifty micrograms total proteins in each lane were separated by 12% SDS-PAGE and transferred onto nitrocellulose membrane. The transformed membrane was blocked for 2 h followed by incubation with primary antibodies at 4 °C overnight. The membrane was washed three times with TBST buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 5 min each and then incubated with 1:200 diluted anti-rabbit or mouse IgG-horseradish peroxidise (HRP) (Thermo Scientific, Rockford, IL) at room temperature for 1 h. The protein band was detected using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

2.11. Cell proliferation assay

$CD4^+$ T cells were purified from spleen of naïve C57BL/6 mice by anti-mouse CD4-beads (Miltenyi Biotec Inc., Auburn, CA). The purified T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and then were transfected with 40 nM Cy5-labeled siMS4a4B2 (Xu et al., 2010) or Cy5-labeled siLuciferase (siLuc) as control. After siRNA transfection, 2×10^5 cells were distributed in 24 well plates pre-coated with anti-CD3 (5 μg/ml)/anti-CD28 antibodies (2 μg/ml) for stimulation. T cell proliferation was assessed by flow cytometric analysis according to CFSE intensity.

2.12. Statistical analysis

Statistical difference between groups was determined by Student's *t* test. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Enhanced expression of MS4a4B in TCR-activated T cells is associated with down-regulation of T cell proliferation

We previously showed that MS4a4B expression is upregulated in mitogen-activated primary T cells (Xu et al., 2006). To test whether MS4a4B expression in T cells can be enhanced by activation signals through TCR, we stimulated primary T cells from mouse spleens with anti-CD3/anti-CD28 antibodies and examined expression of MS4a4B protein by western blotting with anti-MS4a4B antibody. We found that while MS4a4B was expressed in unstimulated T cells, its expression was markedly enhanced at 24 h after stimulation (Fig. 1A). To determine whether enhanced MS4a4B expression in activated T cells inhibits T cell proliferation, we knocked down MS4a4B expression in anti-CD3/anti-CD28 activated T cells by siRNA (siMS4a4B). The results showed that transfection of activated T cells with siMS4a4B decreased MS4a4B expression (Fig. 1B and C). We analyzed proliferation of

siRNA-transfected T cells by flow cytometry. Consistent with our previous observation, knockdown of MS4a4B expression markedly enhanced T cell proliferation (Fig. 1D).

3.2. Cloning and Identification of cis regulatory elements in mouse MS4a4B promoter

We next dissected the mechanism underlying TCR stimulatory signal-induced MS4a4B expression. Given that TCR stimulation upregulates MS4a4B expression in T cells, we postulated that TCR stimulatory signals induce transactivation of unknown transcription factors which bind to the MS4a4B promoter and enhance MS4a4B gene transcription and protein production. To test this hypothesis, we cloned a 2495 base pair (bp) sequence (GenBank ID: HQ585958) in 5′-flanking region upstream of the translation start code of the MS4a4B gene from genomic DNA extracted from C57BL/6 mice (Fig. 2A). To identify regulatory elements in MS4a4B promoter region, we generated a series of MS4a4B promoter fragments with different length truncation in the 5′-terminus, beginning at various upstream positions (between −2495 and −70) and ending just before the translation start site (position +1) by PCR amplification (Fig. 2B). These fragments were inserted into the promoterless pGL4.20 vector, upstream of the luciferase reporter gene to generate serial promoter-reporter constructs (P1–P9 in Fig. 2B). To determine regulatory domains in the promoter region, we used these constructs to transfect EL4 thymoma cells and a T cell line (T32 cells). Promoter activity in the truncated fragments was determined by the expression of reporter luciferase gene. We obtained similar results from the two different types of cell lines. We identified at least two potential activator binding regions and two potential repressor binding regions. The activator binding sites were localized to two fragments, a 442-bp region (region A) positioned from −1176 to −735, and a 119-bp region (region B) positioned from −188 to −70 (Fig. 2C). We analyzed transcription factor binding sites in the activation regions of the MS4a4B promoter by using MatInspector, a software tool for promoter analysis (Cartharius et al., 2005). MatInspector analysis showed that region A contains a consensus binding motif of AP-1 family of transcription factors (aatGAGTaagt) (Fig. 2C).

3.3. AP-1 binding site in the MS4a4B promoter has a role in the regulation of promoter activity

To confirm whether the AP-binding site in the MS4a4B promoter plays a role in regulating promoter activity, we performed site mutation in AP-1 binding sequence of P3-report plasmid to generate a mutant construct ($P3^{AP1mut}$) (Fig. 3A). To test the impact of AP-1 site mutation on MS4a4B promoter activity, T32 cells were transfected with $P3^{AP1mut}$ construct or $P3^{AP1}$ construct as control. Cells lysates were harvested at 48 h after transfection for measurement of promoter activity by luciferase activity assay. The results showed that mutation on the AP-1 site significantly decreased MS4a4B promoter activity (Fig. 3B).

3.4. TCR stimulation upregulates expression of AP-1 transcription factor and enhances its binding to the MS4a4B promoter

It has been shown that AP-1 transcription factors bound to the AP-1 site can activate gene transcription and enhance protein translation (Shaulian and Karin, 2002; Vogt, 2001). To investigate the possibility that AP-1 may play a role in TCR-stimulation-induced upregulation of MS4a4B, we examined expression of cellular Jun (c-Jun) and cellular Fos

(c-Fos) in T cells during TCR-mediated activation. We stimulated primary T cells from spleens in culture with anti-CD3 and anti-CD28 antibodies. Cell samples were harvested from culture at serial time points for detection of c-Jun, c-Fos and MS4a4B mRNA expression by real-time quantitative PCR. The results showed that mRNA expression of c-Jun and c-Fos reached a peak at 3 h of stimulation (Fig. 4A and B), which followed by an increased MS4a4B transcription (Fig. 4C). The enhanced levels of c-Jun and c-Fos in lysates of cell samples were also confirmed by western blotting with anti-c-Jun or c-Fos antibodies respectively. The results showed that levels of c-Jun and c-Fos proteins were increased at 16 h and 12 h respectively after TCR-stimulation (Fig. 5A).

To determine whether TCR stimulation-induced AP-1 protein binds to the MS4a4B promoter, we performed Electrophoretic Mobility Shift Assays (EMSA). We isolated nuclear protein from anti-CD3/anti-CD28-activated spleen cells at 24 h after stimulation. Binding of nuclear protein to the AP-1 consensus sequence was detected by a biotin-labeled probe that contains AP-1 consensus binding sequence. An unlabeled probe containing the same sequence was used for competition assay to confirm the specificity of the probe. The results showed that nuclear protein from TCR-stimulated T cells bound to the labeled probe for AP-1 binding motif and this binding was blocked by co-incubation with unlabeled AP-1 binding DNA fragment but not mutated AP1 probes. Moreover, addition of anti-c-Jun antibody to the binding reaction induced a supershift of AP-1 probe-protein complex. These data indicate that TCR-induced AP-1 transcription factors are capable of binding to the AP-1 site in the MS4a4B promoter (Fig. 5B).

3.5. TCR-stimulation-induced AP-1 binds to the MS4a4B promoter and enhances MS4a4B expression in T cells

We performed ChIP assay to further confirm whether AP-1 binds to MS4a4B promoter region of chromatin in T cells during T cell activation. We isolated nuclear proteins from anti-CD3/anti-CD28-activated T cells. AP-1-associated chromatins in nuclear extract were immunoprecipitated with anti-c-Jun-coupled beads. To determine whether AP-1 is associated with the MS4a4B promoter, c-Jun-associated chromatin was used as template for PCR amplification with primers for the MS4a4B promoter. The results showed that anti-c-Jun antibody specifically pulled down the AP-1-associated DNA sequence of the MS4a4B promoter (Fig. 6A and B). To determine whether AP-1 plays a role in upregulating MS4a4B protein expression, we stimulated primary T cells with anti-CD3/anti-CD28 antibodies in the presence of AP-1 inhibitor. Our data showed that addition of AP-1 inhibitor in culture markedly reduced MS4a4B expression in T cells (Fig. 6C).

4. Discussion

Inhibitory roles of MS4A proteins, e.g. HTm4 and MS4a4B, have been documented by others and our own studies (Chinami et al., 2005; Donato et al., 2002). Donato et al. found that HTm4 protein is expressed in hematopoietic cells and that expression of HTm4 inhibits cell cycle progression in these cells (Donato et al., 2002). Similar to HTm4, we found in our previous studies that MS4a4B expression in T cells is up-regulated upon T cell activation and that up-regulated MS4a4B functions as a modulator to reduce T cell proliferation (Xu et al., 2006, 2010). It appears that MS4a4B is involved in regulation of T cell proliferation as a

negative feedback modulator. However, it is not clear how T cell activation facilitates MS4a4B expression. In this study, we report the MS4a4B promoter for the first time and demonstrate that TCR stimulation-induced AP-1 transcription factor binds to an activation motif in the MS4a4B promoter and enhances production of MS4a4B protein. Our data provide an evidence for the missing link of MS4a4B-mediated negative feedback regulatory loop in T cells and support the notion that MS4a4B serves as a negative modulator in maintenance of physiological balance in the immune system by preventing activated T cells from over-reaction.

AP-1 is a protein family that can bind to a common DNA binding site to regulate activation of many gene transcription (Rauscher et al., 1988b; Shaulian and Karin, 2002). AP-1 complex consists of dimers of primarily the Jun and Fos subfamily members, such as c-Jun and c-Fos (Rauscher et al., 1988a; Sassone-Corsi et al., 1988). It has been shown that activation of the mitogen-activated protein kinase (MAPK) pathway can induce transactivation of AP-1 proteins (Weiss and Bohmann, 2004). c-Jun, a member of the Jun subfamily, is the first oncogene that has been identified as a transcription factor (Woodgett, 1990). c-Fos cannot homodimerize. However, c-Fos can dimerize with c-Jun to form stable heterodimers and enhance their DNA binding ability. Data from the studies in the last few decades confirm that c-Jun and c-Fos are extensively involved in regulation of proliferation, transformation, apoptosis and survival of cells (Shaulian and Karin, 2001). The function of c-Jun is largely dependent on cell type, stimulatory signals, and the microenvironment in cells. In most cases, c-Jun is a positive regulator for cell proliferation and apoptosis (Shaulian and Karin, 2002). However, it may play a protective role and promote cell survival under certain circumstance (Rebollo et al., 2000). In our study, we found that expression of c-Jun and c-Fos is markedly enhanced at 16 h and 12 h respectively after TCR stimulation. This temporal expression of c-Jun and c-Fos occurs immediately before upregulation of MS4a4B protein in T cells as we observed previously (Xu et al., 2006, 2010), suggesting that c-Jun transactivation may be at least partially responsible for the enhanced MS4a4B expression. Thus, our data raise the possibility that AP-1, in addition to its positively regulatory effects, may play an inhibitory role in activated T cells by upregulating MS4a4B expression. Our data do not exclude the possibility that other transcription factors and repressors may also be involved in regulation of MS4a4B expression during T cell activation given that we also identified, by MatInspector analysis, a few other transcription factor and repressor binding sites in the MS4a4B promoter (Fig. 2B and C). Given that AP-1 binding motif is only found in the region A (−1176 to −735) but not in the region B (−188 to −70) of the MS4a4B promoter and mutation of the AP-1 binding site only partially reduces activity of the MS4a4B promoter, the region B of the MS4a4B promoter likely uses other transcription factor but not AP-1 for activation. Therefore, the potential roles of other transcription factors and repressors in regulation of MS4a4B protein production remain to be investigated.

T cells become activated and proliferate in response to exposure to various antigens or stimuli. This reaction of T cells usually tends to be “self-limited” after antigens or stimuli are removed. It has been shown that anti-proliferative signals play roles in maintaining physiological balance of the immune system (Green et al., 2003; Rubtsov and Rudensky, 2007). The data presented in this article, together with our previous findings (Xu et al.,

2010), indicate that MS4a4B is one of important regulatory proteins that are involved in regulation of T cell proliferation during TCR-induced immune responses. We propose that MS4a4B plays a role in the negative feedback regulatory loop in activated T cells: TCR stimulatory signals induce T cell proliferation and promote immune responses to the stimuli; on the other hand, the same stimulation also induces AP-1-mediated upregulation of MS4a4B, which inhibits the proliferation of activated T cells to prevent their over-reaction. In this model, whether activated T cells exit from cell cycle to arrest, or continue to proliferate is largely dependent on the strength of stimuli and inhibitory signals. Therefore, continuous stimulation by excessive antigen exposure may override regulatory effects of MS4a4B (and other negative regulators as well).

5. Conclusions

In this study, we cloned and analyzed the MS4a4B promoter. We found that TCR stimuli-induced AP-1 transcription factor binds to AP-1 motif in the MS4a4B promoter and facilitates MS4a4B expression in activated T cells. The elevated expression of MS4a4B protein subsequently inhibits proliferation of activated T cells. These data indicate that TCR stimulation induces upregulation of MS4a4B protein at least partially through AP-1 transcription factor. Thus, TCR-stimulation→AP-1→MS4a4B↑→T cell proliferation ↓ axis constitutes an important negative feedback regulatory mechanism during T cell immune responses.

Acknowledgments

The authors thank Mrs. Katherine Regan, Department of Neurology, Thomas Jefferson University, for editorial assistance. This work was supported by grants from the American Heart Association (0830072N) (H.X.).

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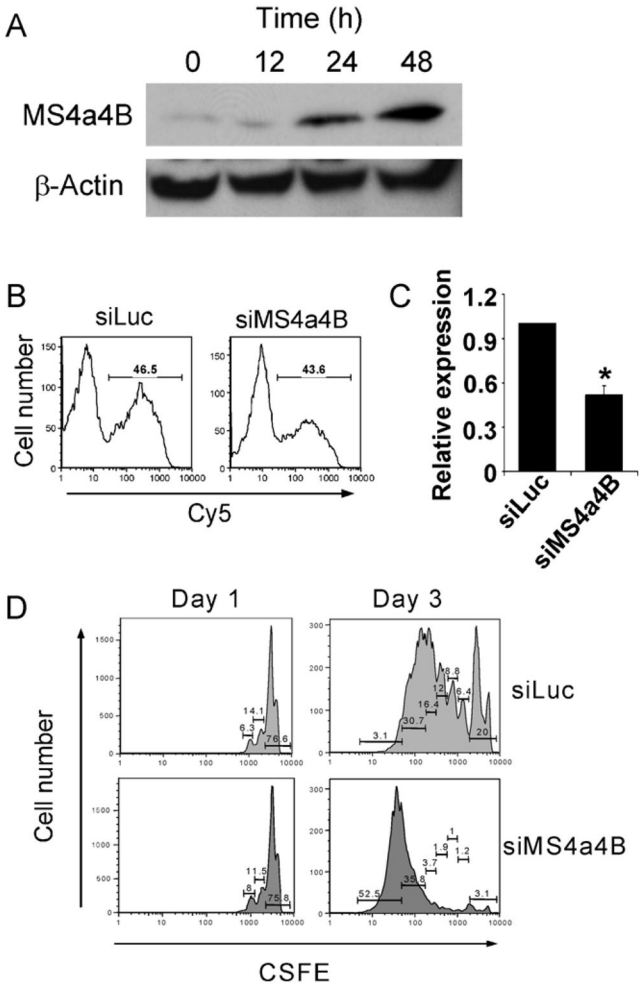

Fig. 1.

MS4a4B expression is associated with reduced T cell proliferation. (A) The primary T cells were treated on 24 well plate coated with anti-CD3 (5 μg/ml)/anti-CD28 (2 μg/ml) antibodies for 12, 24 and 48 h respectively. Cells harvested from culture were lyzed by lysis buffer. Total lysates were then analyzed by western blotting with anti-MS4a4B antibody and anti-β-Actin antibody. One representative of two independent experiments is shown. (B) Purified T cells pre-labeled with CFSE were stimulated with anti-CD3 and anti-CD28 antibodies as described in "A" and transfected twice with either Cy5-labeled si-MS4a4B or Cy5-labeled si-Luc siRNAs (12 h rest between transfections). Efficiency of siRNA transfection in cells was determined by flow cytometry based on percentage of $Cy5^+$ cells. (C) MS4a4B RNA expression in siRNA-transfected cells (including $Cy5^+$ and $Cy5^-$ cells) were analyzed by real-time quantitative PCR. The results are presented as SEM of triplicates. Statistical significance was determined by *t*-test. *, $P < 0.05$. (D) Cell proliferation was analyzed on day 1 and day 3 after the 2nd transfection by flow cytometry according to dilution of CFSE fluorescence (FL1) in $Cy5^+$ gate (FL4). Percentage of cells in each generation was shown in histograms. One representative of three independent experiments is shown.

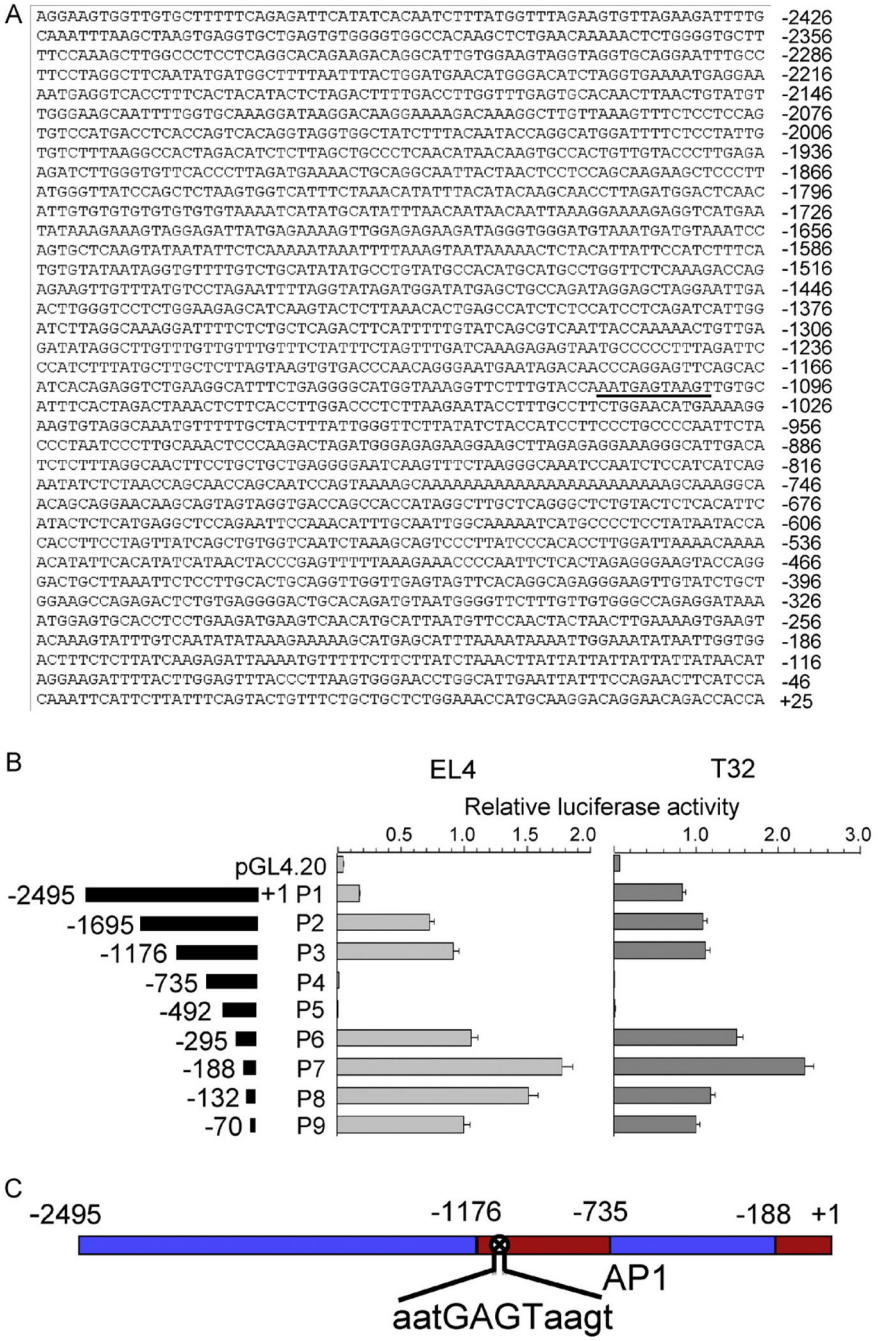

Fig. 2.

Identification of *cis*-regulatory elements in MS4a4B promoter region. (A) Nucleotide sequence of the MS4a4B promoter. The promoter region of MS4a4B gene was cloned from C57BL/6 mice. The predicted AP-1 binding site is underlined. (B) Deletion analysis of MS4a4B promoter activity in EL4 and T32 cell lines. A series of fragments from the 5′-flanking MS4a4B promoter region were cloned into pGL4.20 report plasmids. Each promoter-reporter construct (or a promoterless pGL4.20 as control) was co-transfected with pRL-SV40 into EL4 and T32 cells. Luciferase activities were measured after 48 h and

normalized according to transfection efficiency. Promoter activity was assessed by relative luciferase activity of each construct (tested construct vs. P9 construct). Data are shown as mean ± SE of three independent experiments. (C) Schematic representation of transcription activation regions (red) and repression regions (blue) in the MS4a4B promoter. Core sequence of AP-1 binding site is shown.

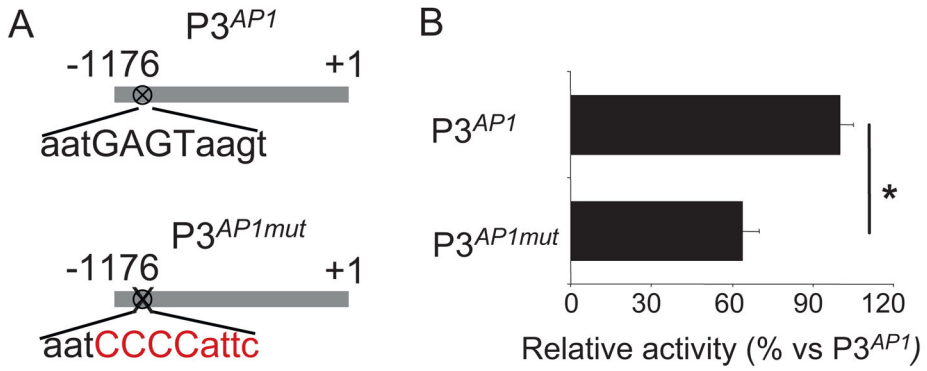

Fig. 3.

Mutation at AP-1 binding site in the MS4a4B promoter decreases the promoter activity. (A) Wild type ($P3^{AP1}$) and mutant ($P3^{AP1}$) reporter constructs. Mutation of AP-1 binding sequence was generated by PCR cloning. (B) T32 cells were transfected with $P3^{AP1}$ or $P3^{AP1mut}$ constructs. Cell samples were harvested and lyzed. Luciferase activity in cell lysates was measured as described in Section 2. The experiments were performed in triplicate for each sample and were repeated three times. Data are presented as mean ± SD of % relative luciferase activity vs. the $P3^{AP1}$ construct. Statistical significance was determined by *t*-test. *, $P < 0.05$.

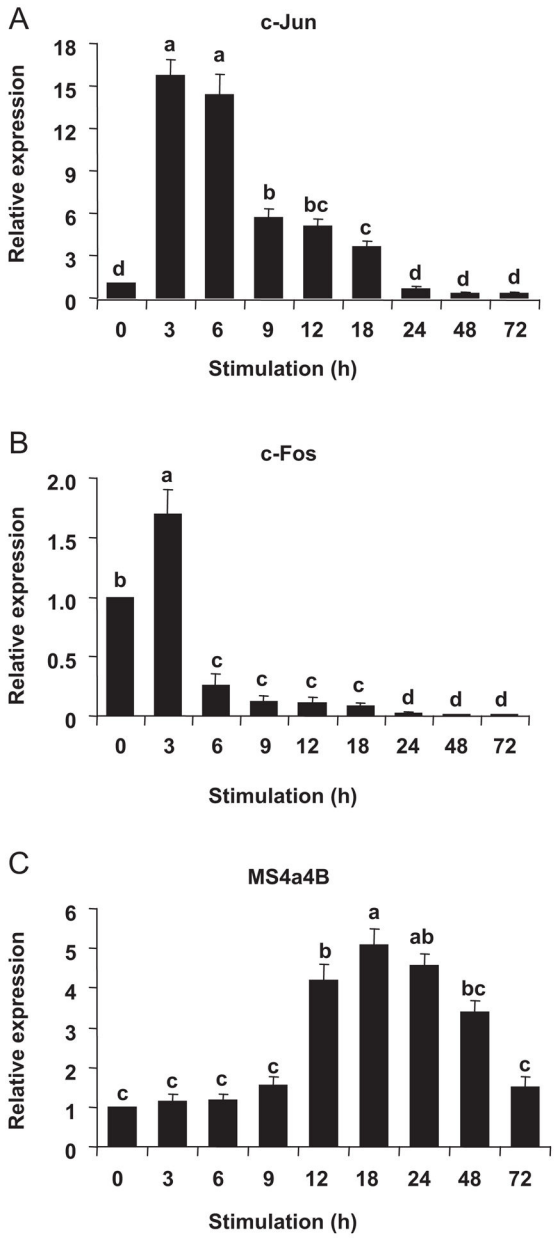

Fig. 4.

TCR stimulation-induced transcription of c-Jun and c-Fos is followed by an increased RNA expression of MS4a4B. The primary T cells were stimulated with soluble anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml). Cells were collected at indicated time points after stimulation. Total RNA was extracted from cells and was transcribed into cDNA. Levels of mRNA for c-Jun (A), c-Fos (B) and MS4a4B (C) were detected by real-time RT-PCR with specific primer pairs for c-Jun, c-Fos and MS4a4B respectively. Statistical significance between time points was determined by *t*-test. *P* values of less than 0.05 were considered as statistical difference and were shown by different letters. One of two experiments is shown.

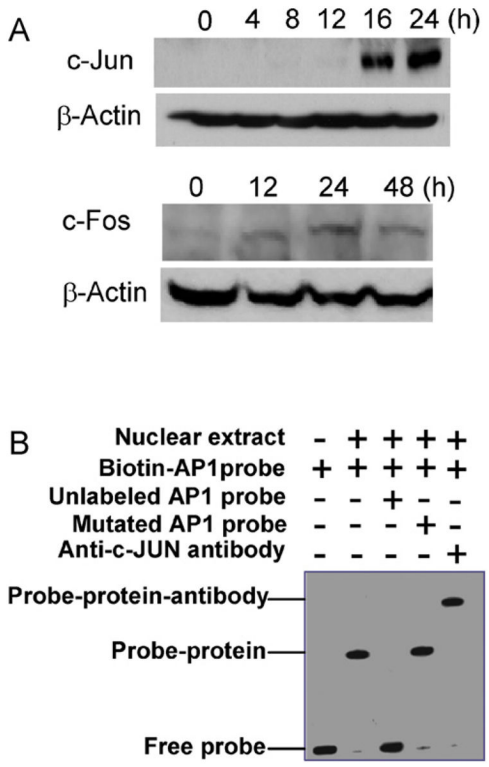

Fig. 5.

TCR stimulation-induced c-Jun specifically binds to AP-1 motif of the MS4a4B promoter. (A) TCR stimulation increased expression of c-Jun and c-Fos. The primary T cells were stimulated on 24 well plate coated with anti-CD3 (5 μg/ml)/anti-CD28 (2 μg/ml) antibodies. Cell samples were harvested at the time points indicated and were lyzed by lysis buffer. Levels of c-Jun and c-Fos proteins in cell lysates were analyzed by western blotting with anti-c-Jun or anti-c-Fos antibodies respectively. One of two experiments with the similar results is shown. (B) TCR-induced transcription factor binds to AP-1 site of the MS4a4B promoter. Nuclear proteins were extracted from anti-CD3/CD28-activated primary T cells at 24 h of stimulation. Binding of nuclear proteins to the AP-1 consensus sequence was determined by EMSA with a double-stranded 20-bp oligonucleotide containing AP-1 as a probe. The labeled AP-1 probe (Lane 1) was incubated with the nuclear extract either alone (Lane 2) or in competition with 200-fold excess amounts of unlabeled probe (Lane 3). Mutated AP1 probe was used as control to confirm the specificity of AP1 probe (Lane 4). Antic-Jun antibody was used to verify c-Jun in the probe-protein complex (Lane 5). One of three experiments is shown.

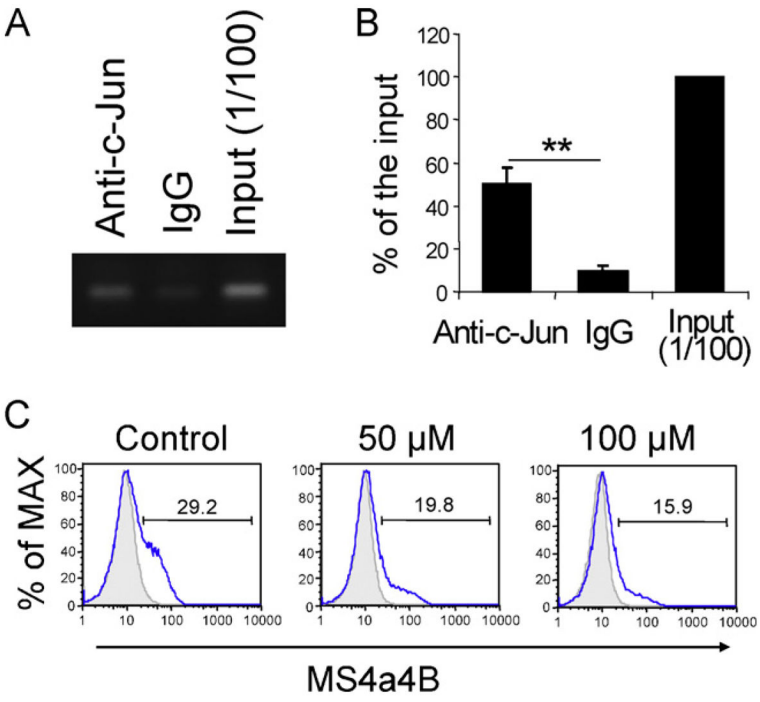

Fig. 6.

TCR stimulation-induced c-Jun enhances MS4a4B expression in T cells. (A) ChIP assay. Nuclear proteins were extracted from anti-CD3/anti-CD28-activated primary T cells at 24 h of stimulation. Association of nuclear proteins with MS4a4B promoter region was determined by ChIP assay. For ChIP assay, AP1-chromatin complex was extracted from nuclear proteins by anti-c-Jun antibody-coupled Protein A-agarose beads. IgG-coupled Protein A-agarose beads were parallel used as a negative control. The MS4a4B promoter sequence in the complex was determined by quantitative PCR amplification of the sequence between −1100 and −900 of the MS4a4B promoter. One of three experiments is shown. (B) Intensity of the bands from PCR experiments described in “A” was determined by densitometry. The results are presented as percentage of input (mean ± SD) from three experiments. Statistical significance between samples was analyzed by *t*-test (**, $P < 0.01$). (C) Inhibition of AP-1 partially reduced MS4a4B expression in T cells. Primary T cells were stimulated with anti-CD3/anti-CD28 antibodies for 3 days in the presence or absence of the AP-1 inhibitor Tashinone IIA. MS4a4B expression in T cells was determined by flow cytometry with anti-MS4a4B antibody (solid line). Normal rabbit IgG was used as isotype control (gray peak). One of two experiments is shown.

Table 1

PCR primers for cloning of reporter constructs with truncated MS4a4B promoter.

Reporter plasmid Sense	Sequence (5′→3′)	Position
P1	CCGGATCCAGGAAGTGGTTGTGCT	−2495→−2480
P2	CCGGATCCTTGGAGAGAAGATAGGC	−1695→−1679
P3	CCGGATCCGGAGTTCAGCACATCAC	−1176→−1160
P4	CCGGATCCCAAGCAGTAGTAGGTGA	−735→−719
P5	CCGGATCCCCAATTCTCACTAGAGC	−492→−476
P6	CCGGATCCCATGCATTAATGTTCCAA	−295→−278
P7	CCGGATCCGTGGACTTTCTCTTATC	−188→−172
P8	CCGGATCCTTATTATTATTATAACATA	−132→−114
P9	CCGGATCCTGAATTATTTCCAGAACT	−70→−52
Anti-sense	TCCTCGAGGTTTCCAGAGCAGCAGC	−1→−17