Available online at www.sciencedirect.com
ScienceDirect
Molecular Immunology 45 (2008) 2028–2037
Molecular Immunology
www.elsevier.com/locate/molimm

Protein phosphatase subunit G5PR that regulates the JNK-mediated apoptosis signal is essential for the survival of CD4 and CD8 double-positive thymocytes

Yan Xing [a,1], Xiaodan Wang [a,1], Hideya Igarashi [a], Hiroshi Kawamoto [b], Nobuo Sakaguchi [a,*]

[a] *Department of Immunology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan*[2]
[b] *Laboratory for Lymphocyte Development, RIKEN Research Center for Allergy and Immunology, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan*

Received 9 September 2007; received in revised form 11 October 2007; accepted 12 October 2007
Available online 19 November 2007

Abstract

Early T lineage cells are selected in the thymus by the specific recognition of peptide components presented by MHC molecules on the surface of thymic epithelial cells and dendritic cells. As a potential regulator of the apoptotic and survival signals, the protein phosphatase 2A-component G5PR regulates Bim phosphorylation in B-cells. Here, we studied whether G5PR is involved in the regulation of the similar apoptotic pathway for cell survival during the selection of thymocytes. T-cell-specific G5PR knockout ($G5pr^{-/-}$) mice displayed thymic atrophy, significant reduction in thymocyte numbers, particularly a 10-fold decrease in the number of CD4 and CD8 double-positive (DP) thymocytes and few mature single-positive (SP) cells. $G5pr^{-/-}$ thymocytes exhibited normal potential of proliferation and differentiation during the transition from double-negative (DN) to DP stage, but significantly increased susceptibility to apoptosis at the DP stage. G5PR deficiency did not affect on Bim activation in thymocytes, but caused hyper-activation of JNK and Caspase-3 with augmented Fas ligand (FasL) expression, indicating that G5PR regulates the thymocyte unique apoptotic signal involved in JNK-mediated Caspase-3 activation but not in Bim activation. G5PR is essential for the survival of DP cells during thymocyte development.

Keywords: T cells; Apoptosis; JNK; Bim

1. Introduction

T-lineage cells are generated during early development with *TCR* rearrangements and expression of T-cell markers in the cortex of the thymus. Early T-cell progenitors with a CD4 and CD8 double-negative (DN) phenotype undergo rearrangement of the *TCRβ* locus and differentiate from DN3 to DN4 thymocytes expressing the pre-T-cell receptor (pre-TCR) (Michie and Zuniga-Pflucker, 2002). Pre-TCR signaling induces cell proliferation and survival, and further stimulates the transition of DN thymocytes to the CD4 and CD8 double-positive (DP) stage (Hoffman et al., 1996). At DP stage, the *TCRa* locus undergoes rearrangements until positive selection (Starr et al., 2003). The productive rearrangement of *TCRα* gene allows DP thymocytes expressing mature TCR$\alpha\beta$. Thus, DP thymocytes, which account for a majority of the cells in the thymus, would be selected as T-cells with the conventional primary TCR$\alpha\beta$ repertoire. Only a minority of DP thymocytes is thought to receive appropriate signals for survival and then differentiate into mature CD4-single-positive (SP) or CD8-SP T-cells; whereas, the majority of thymocytes (90–95%) will die in the negative and positive selection processes by apoptosis, ensuring that useless and potentially self-reactive T-cells do not enter the peripheral pool of T-cells (Palmer, 2003; Starr et al., 2003).

Abbreviations: AICD, activation-induced cell death; DiOC6, 3,3′-dihexyloxacarbocynine iodide; DN, double-negative; DP, double-positive; FasL, Fas ligand; $G5pr^{-/-}$, T-lineage specific G5PR knockout; $G5pr^{f/f}$, floxed *G5pr*; PI, propidium iodide; PP2A, protein phosphatase 2A; SP, single-positive.

* Corresponding author. Tel.: +81 96 373 5135; fax: +81 96 373 5138.
E-mail address: nobusaka@gpo.kumamoto-u.ac.jp (N. Sakaguchi).

[1] These authors contributed equally to this work.

[2] CREST project from JST Japan.

0161-5890/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2007.10.028

TCR recognition of self-ligands in the complex of MHC molecules leads to the stimulation of DP cells through activation of Src-family kinases, including the tyrosine kinase Lck, which phosphorylates the ITAMs of the TCR/CD3ζ chains, then providing docking sites for Syk-family kinases such as ZAP70 (Ashe et al., 1999; Palacios and Weiss, 2004). Phosphorylation of ZAP70 by Lck enables ZAP70 to phosphorylate the trans-membrane adapter molecule LAT. This initiates a cascade of recruitment and activation of further signaling intermediates, including protein kinase C (PKC) and PI3K, intracellular calcium mobilization and activation of one or several MAPK cascades (Acuto and Cantrell, 2000; Berg and Kang, 2001; Horejsi et al., 2004). Many of the molecules associated with TCR$\alpha\beta$ signaling are also involved in pre-TCR signaling, giving rise to the activation of the Lck-ZAP70-MAPK signal transduction pathway (Cantrell, 2002; Muljo and Schlissel, 2000).

Studies of gene-deficient or gene-transgenic mice with the impaired functions of molecules involved in the signal transduction pathways of TCR$\alpha\beta$ and pre-TCR signaling have demonstrated the involvement of MAPK pathways in the decision of whether a DP thymocyte is to live or die. The Ras/Raf-ERK pathway is required for positive selection as shown in the mice with dominant-negative versions of Raf, Ras and MEK, or deficient in ERK1 or Ras-GRP (Alberola-Ila et al., 2003). By contrast, JNK and p38 pathways have been implicated in negative selection. *TCR* transgenic mice expressing a dominant-negative *JNK1* transgene showed a decreased level of apoptosis after treatment with a negative-selecting peptide, and JNK2-deficient thymocytes were resistant to apoptosis induced by cross-linking of CD3 (Rincon et al., 1998; Sabapathy et al., 1999). The blocking of p38 activation with pharmacological reagents interfered with negative, but not positive selection in the fetal thymus organ culture (Sugawara et al., 1998). The activities of MAPK members are regulated by various phosphatases in cells of various tissues and organs (Tamura et al., 2002); however, the detailed molecular mechanism underlying the dephosphorylation of MAPKs during thymocyte development remains largely undetermined. Protein phosphatase 2A (PP2A) appears to be the major phosphatase in eukaryotic cells that down-regulates the activation of protein kinases (Anderson et al., 1990; Gomez and Cohen, 1991; Millward et al., 1999). PP2A is a prevailing negative regulator of JNK in the context of an inflammatory stimulus (Shanley et al., 2001).

G5PR, a B$''$ regulatory subunit of PP2A, is inducibly expressed in mature B-cells after BCR cross-linking *in vitro* (Huq Ronny et al., 2006; Kono et al., 2002). B-cell-specific G5PR knockout mice displayed a significantly decreased number of mature B-cells, and G5PR-deficient B-cells showed enhanced activation of JNK upon BCR cross-linking, leading to an increase in Bim activation and depolarization of the mitochondrial membrane, and increased susceptibility to BCR-mediated apoptosis (Xing et al., 2005). The observation that G5PR is expressed abundantly in the thymus (Kono et al., 2002) suggests its association with survival and the positive or negative selection process for thymocytes. Here, we prepared T-lineage specific G5PR knockout mice ($G5pr^{-/-}$) by mating floxed *G5pr* and *Lck-Cre* transgenic mice. $G5pr^{-/-}$ mice display increased activation of JNK in thymocytes and a marked decrease in the number of the DP and SP populations, suggesting a critical role for G5PR-associated phosphatase in the regulation of JNK activation during early T-cell differentiation in the thymus.

2. Materials and methods

2.1. Mice

Generation of $G5pr^{F/F}$ mice has been described previously (Xing et al., 2005). To delete the neo gene, we bred female $G5pr^{F/F}$ mice with male *EIIa-cre* transgenic mice (Lakso et al., 1996), and obtained $G5pr^{f/wt}$ mice carrying two *Lox-P* sites flanking the *Exon 1* and *Exon 2* of the *G5pr* gene. $G5pr^{f/wt}$ mice were maintained on a C57BL/6 background. T-cell-specific G5PR-deficient ($G5pr^{-/-}$) mice were generated by crossing $G5pr^{f/f}$ mice with *Lck-Cre* trangenic mice, in which the *Cre* gene is under the control of the *p56Lck* proximal promoter (Takahama et al., 1998). All mice were maintained in the Center for Animal Resources and Development (CARD), Kumamoto University. Experimentation and animal care were in accordance with the guidelines of the CARD in Kumamoto University.

2.2. RT-PCR

Total RNA purified with RNeasy Mini Kit (Qiagen) was used to synthesize cDNA for PCR assay. For *G5pr* expression, the following primers from *Exon 3* and *Exon 15* of *G5pr* were used: 5′-TAT AAG ACC ATA CCC CGG TTT TAT TAC AGG-3′ and 5′-TCA TGT GTC ATC AAG ATC TGC AGA GTT CTC-3′. For *Actb*, 5′-CCT AAG GCC AAC CGT GAA AAG-3′ and 5′-TCT TCA TGG TGC TAG GAG CC-3′ were used. For *Fasl*, 5′-CTA CCA CCG CCA TCA CAA C-3′ and 5′-CCT CTT CTC CTC CAT TAG CAC-3′ were used. For *Bcl11b*, 5′-GGT CTT CAA GAA CTG TAG CAA-3′ and 5′-CCG TGC CAC TTT TTC ATG TGT-3′ were used. For *Lef-1*, 5′-AAC TCT GCG CCA CCG ATG AG-3′ and 5′-AGA AAA GTG CTC GTC GCT GT-3′ were used. Other primers include those previously described for *Tcf-1*, *Notch1*, *Hes1* and *Deltex* (Masuda et al., 2005), *c-Myc* (Dose et al., 2006) *ROR*γ, and *ROR*γt (Xi and Kersh, 2004). Cycling times and temperatures were as follows: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. Amplification was performed for 25 cycles for *Actb* and 35 cycles for others. The resolved bands were photographed and quantified with QUANTITY ONE software (Bio-Rad Laboratories). The ratio of *Fasl* mRNA expression to that of *Actb* were calculated.

2.3. Flow cytometric analysis

Single cell suspensions were stained by fluorescent isothiocyanate (FITC)-, phycoerythrin (PE)-, allophycocyanin (APC)- or biotin-conjugated mAbs of anti-CD4, anti-CD8, anti-CD24, anti-CD25, anti-CD44, anti-TCRβ, anti-Fas (BD Biosciences) and streptavidin PE-Cy7 (eBioscience), and analyzed by FACSCalibur using CELLQuest (Becton Dickinson) and Flowjo (Tree Star) softwares. Intracellular staining was carried out