International Journal of Biochemistry and Cell Biology 92 (2017) 95–103

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

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

Research paper

A T cell-specific knockout reveals an important role for protease-activated receptor 2 in lymphocyte development☆

Nidhish Francis[a,1], Alison L. Every[a], Babatunde A. Ayodele[a], Robert N. Pike[b], Eleanor J. Mackie[a], Charles N. Pagel[a,*]

[a] Department of Veterinary Biosciences, Melbourne Veterinary School, University of Melbourne, Parkville, VIC 3010, Australia
[b] Department of Biochemistry & Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, VIC 3086, Australia

ARTICLE INFO

Keywords:
Protease-activated receptors
T cells
Cell differentiation
Cell surface molecules
Knockout mice
Thymus

ABSTRACT

Activation of protease-activated receptor-2 (PAR_2) expressed by T cells has been linked to the bone loss associated with periodontitis. We generated PAR_2 conditional-null mice and crossed these with mice expressing Cre recombinase under control of the Lck proximal promoter, to produce T cell-specific PAR_2-null mice in order to further study the cellular mechanism involved in periodontitis. Here we report that efficient deletion of PAR_2 in thymocytes isolated from T cell-specific PAR_2-null mice resulted in thymic and splenic hypoplasia and a reduction in the cells of the cortex and a loss of distinction between the cortex and the medulla of the thymus. FACS analysis confirmed significant reductions in CD4 and CD8 double negative (DN3 and DN4) sub-populations, as well as double positive and single positive T cells, in T cell-specific PAR_2-null mice compared to Cre expressing PAR_2 wild-type mice. The proportion of annexin V positive and propidium iodide negative cells was increased in CD4 and CD8 double negative, double positive and single positive T cells from T cell-specific PAR_2-null mice. No change in the proportion of Ki67 positive cells was observed in sections of thymus from T cell-specific PAR_2-null mice, suggesting that the depletion of T cell sub-populations in T cell-specific PAR_2-null mice resulted from increased apoptosis rather than reduced proliferation. Together, these results demonstrate that PAR_2 plays an important and previously unrecognised anti-apoptotic role in T cell development and suggest that the PAR_2 conditional-null mouse will be an important resource for determining tissue and cell specific effects of PAR_2.

1. Introduction

Protease-activated receptors (PARs) are members of a sub-group of the seven transmembrane domain G protein-coupled receptor family that are activated by proteolytic cleavage of their amino terminal extracellular domain, rather than by occupancy of the receptor by its ligand (Adams et al., 2011). Of the four members of the family, PAR_2 is widely expressed, is activated by a broad range of trypsin-like proteases and has been implicated in many physiological and pathological processes, including inflammation, fibrosis, nociception and tumour initiation and progression (Adams et al., 2011; Kularathna et al., 2014; Nguyen et al., 2003; Rothmeier and Ruf, 2012; Vergnolle et al., 2001).

Periodontitis is a chronic inflammatory disease associated with the destruction of the periodontal tissues, including bone, leading to tooth loss; it is also associated with systemic conditions including cardiovascular disease and diabetes (Williams et al., 2008). A major aetiological agent of periodontitis is the obligate anaerobic bacterium *Porphyromonas gingivalis* (Haffajee and Socransky, 1994). The gingipains are three cysteine proteases expressed by *P. gingivalis* that act as major virulence factors and are capable of activating PAR_2 and inducing pro-inflammatory responses from mammalian cells *in vitro* (Lourbakos et al., 1998; Lourbakos et al., 2001). The pivotal role of activation of PAR_2 in the development of destructive bone loss associated with *P. gingivalis*-induced periodontitis *in vivo* was demonstrated by Holzhausen et al. using global PAR_2 knockout mice (Holzhausen et al., 2006).

Strong evidence exists that implicates T cells in the progression of

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DN, double negative; FSC-A, forward-scattered light area; FSC-H, forward-scattered light height; *Lck-Cre::F2rl1*$^{w/w}$, Lck-Cre positive wildtype mice; *F2rl1*$^{fl/fl}$, Lck-Cre negative PAR_2 floxed mice; *Lck-Cre::F2rl1*$^{fl/fl}$, Lck-Cre positive PAR_2 floxed mice (T cell specific PAR_2 null mice); MNE, mean normalised expression; PAR, protease-activated receptor; qPC, Rquantitative PCR; SSC-A, side-scattered light area

☆ This work was supported by the National Health and Medical Research Council [project grant number 1044103].

* Corresponding author.
E-mail address: cpagel@unimelb.edu.au (C.N. Pagel).
[1] Present address: School of Animal and Veterinary Sciences, Charles Sturt University, Wagga Wagga, NSW 2650, Australia.

http://dx.doi.org/10.1016/j.biocel.2017.09.015
Received 1 June 2017; Received in revised form 19 September 2017; Accepted 21 September 2017
Available online 22 September 2017
1357-2725/

Table 1
Sequence of PCR oligonucleotide primers.

Name	*Forward (5′–3′)*	*Reverse (5′–3′)*
F2rl1 (genotyping)	P1 CTGCCTTACTCACCAAAGACTCG	P2 CTTCTTCCTTTACTGTTGTTGC P3 CAACGGGTTCTTCTGTTAGTCC
LoxP	CCGGAACCGAAGTTCCTATT	CAAGGCCAGGTGTAGATTCC
PAR_2 Exon 2	TCTCTGCACCAATCACAAGC	GCAAGTCACTGACCCTCCAT
Lck Cre	AACCCAGTCAGGACTTGAA	GGCAACACCATTTTTTCTGACC
GAPDH	TTACTCCTTGGAGGCCATGT	TAAGAGCAACTGGGGGTTTG

the disease. For example, CD4-null mice have less bone loss in experimental periodontitis than wild-type mice (Baker et al., 1999). Furthermore, our group has shown that global deletion of PAR_2 results in reduced T cell activation and bone loss in mice immunized or infected with *P. gingivalis* (Wong et al., 2010), suggesting that activation of PAR_2 contributes to the progression of periodontitis by affecting the activation and differentiation of T cells. Although it appears that T cells are important for the progression of periodontitis, other cell types such as oral epithelial cells, neutrophils and mast cells are likely to be involved in the disease, and many of these cells are known to express PAR_2 (Wong et al., 2010). As the two studies regarding the involvement of PAR_2 in the pathogenesis of periodontal disease undertaken so far have used mice in which the PAR_2 gene was knocked out in all cells (global PAR_2-null), it is not possible to determine from these studies which of the PAR_2-expressing cell types was or were responsible for the effects observed. In order to understand the mechanism of PAR_2's involvement in periodontal disease, it is critical to identify these cells. Thus, in order to identify the contribution of specific cell types, including T cells, to the immune response of mice *to P. gingivalis* and the resulting bone loss, we derived mice harbouring a conditional knockout allele of the *F2rl1* gene that encodes PAR_2.

In this paper, we describe the derivation of *F2rl1* conditional knockout mice and describe the unexpected phenotype of the lymphocyte-specific PAR_2 null mice that result from crosses with mice expressing Cre under control of the lymphocyte-specific Lck proximal promoter (Lee et al., 2001). The results of this study suggest that PAR_2 plays an important and previously unidentified anti-apoptotic role in T cell development.

2. Materials and methods

2.1. Animals

Embryonic stem cells (JM8A3.N clone EPD0592_3_H01) with a targeted, knockout first mutation in the gene encoding PAR_2 (*F2rl1*) were obtained from the European Mouse Mutant Cell Repository (https://www.eummcr.org/; Skarnes et al., 2011). The mice were generated *via* ES cell microinjection into Balb/c blastocysts to generate chimeric mice, which were bred for germline transmission through the ES Cell to Mouse service of the Australian Phenomics Network (APN) at Monash University (www.australianphenomics.org.au). Male mice expressing FLPe (Farley et al., 2000) were kindly provided by Professor Susan Dymecki (Department of Genetics, Harvard Medical School). These mice were crossed with female mice carrying the PAR_2 knockout out first mutation $F2rl1^{tm1a(EUCOMM)Wtsi}$ allele in order to generate conditional mice carrying the $F2rl1^{tm1c(EUCOMM)Wtsi}$ allele. Transgenic male mice expressing Cre-recombinase under control of the Lck proximal promoter (Lee et al., 2001) were obtained from the Walter and Eliza Hall Institute of Medical Research (Parkville, Vic, Australia) and were crossed with female conditional PAR_2 knockout mice in order to generate T cell-specific PAR_2-null mice. The colony of T cell-specific PAR_2-null mice was maintained by crossing mice positive for Lck-Cre and heterozygous for the floxed allele (*Lck-Cre::*$F2rl1^{fl/w}$), thus all experimental mice were littermates or the offspring of littermate mice.

Global PAR_2 knockout mice [$gPAR_2^{-/-}$; (Lindner et al., 2000)] obtained from Professor Shaun Coughlin (Cardiovascular Research Institute University of California, San Francisco), were maintained as heterozygous breeding stock. The use of animals in this study was approved by the Gene Technology and Biosafety Committee of the University of Melbourne and the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences (AEC 1212655). Mice were housed in a controlled environment with free access to food and water, and all work was conducted in compliance with the Australian Code for the Care and Use of Animals for Scientific Purposes (2013) and the National Health and Medical Research Council's Guidelines for the Generation, Breeding, Care and Use of Genetically Modified and Cloned Animals for Scientific Purposes (2007).

2.2. Polymerase chain reaction

Polymerase chain reactions were performed on template DNA in a 20 µl total reaction volume containing forward and reverse oligonucleotide primers [1 µM each; Geneworks, Hindmarsh, SA, Australia; Table 1 (Pagel et al., 2003)]. Thermal cycling was conducted over 35 cycles (95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s), prior to resolution of amplified products on agarose gels in the presence of SYBR safe (Life Technologies, Grand Island, NY USA).

2.3. Cell purification

Single cell suspensions of thymus and spleen from 6 to 8 week old mice were obtained by disaggregation of the whole organ through a 70 µm cell strainer (BD Bioscience, San Jose, CA, USA). After lysis of red blood cells, the cell suspensions were either washed in FACS buffer (PBS, 2% [v/v] FCS, 1 mM EDTA) and pelleted by centrifugation or were used to purify T cells using Mouse Pan T (CD90.2) magnetic beads (Life Technologies) according to the manufacturer's instructions.

2.4. DNA sequencing

Sequencing of genomic DNA and PCR products was performed on both strands using BigDye Terminators v3.1 (Life Technologies) according to the manufacturer's instructions. Following amplification and ethanol precipitation, sequencing of products was conducted by capillary electrophoresis (Australian Genome Research Facility, Parkville, Vic, Australia). Sequence data files were analysed, aligned and compiled into contiguous sequences using the Serial Cloner 2.6.1 software.

2.5. Calcium mobilisation assays

Mouse primary calvarial osteoblasts were isolated as described (Pagel et al., 2009). Cells were seeded into 96 well plates at a density of 3×10^4 cells/well. Twenty-four hours later, medium was removed and the cells were incubated in assay buffer (HBSS, 0.1% [w/v] BSA and probenecid 2.5 mM [w/v]) containing Fura-2 AM (1 µM [w/v]; Molecular Probes, Life Technologies) and pluronic acid (0.01% [w/v]) for 1 h at 37 °C). Following incubation, cells were washed twice with the assay buffer and incubated in assay buffer. The fluorescent emission at