

PAR-2 Deficient CD4⁺ T Cells Exhibit Downregulation of IL-4 and Upregulation of IFN- γ after Antigen Challenge in Mice

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ABSTRACT

Background: To investigate the functional role of protease activated receptor (PAR) -2 in T lymphocytes, we analyzed TCR-mediated inflammatory cytokine production using PAR-2 deficient (KO) and wild type (WT) mice.

Methods: Production of serum IgE and cytokines by spleen CD4⁺ T cells was determined in OVA-sensitized and OVA-challenged mice of PAR-2 KO in contrast to WT mice. Phosphorylation of JNK1 and 2 was determined by Western blotting.

Results: A reduction in serum levels of IgE and IL-4 production by splenic CD4⁺ T cells from OVA-sensitized and OVA-challenged KO mice compared to WT mice was observed. By contrast, IFN- γ production was upregulated after antigen stimulation in KO mice. Anti-CD3-mediated phosphorylation of JNK1 was upregulated in splenic CD4⁺ T cells from KO mice compared to WT mice.

Conclusions: PAR-2 participates in the regulation of T cell cytokine production that may be caused by modulation of JNK1 phosphorylation.

KEY WORDS

CD4⁺ T cell, GATA-3, JNK1, PAR-2, T-bet

INTRODUCTION

Protease-activated receptors (PARs) are members of the family of seven-transmembrane, G protein-coupled receptors.¹⁻³ Among the 4 PARs identified so far, PAR-1, -3 and -4 are cleaved and activated by thrombin, whereas PAR-2 is preferably cleaved by trypsin, mast cell tryptase, factor VIIa and factor Xa.³⁻⁷ Each of the PARs has been shown to modulate a variety of physiological processes such as cytokine and mediator release, vasodilatation, platelet aggregation, cellular proliferation.⁴ A role for PAR-2 in inflammation has been suggested by demonstration of the fact that PAR-2 mRNA was upregulated by IL-1 α , TNF- α or LPS in cultured human endothelial cells.⁸ *In vivo*,

activation of PAR-2 has been shown to induce various inflammatory responses such as increased leukocyte rolling and adherence in mesenteric venules,⁹ leukocyte recruitment into the peritoneal cavity,⁹ edema formation and granulocyte infiltration in the rat paw.^{10,11} Studies performed using PAR-2-deficient mice have demonstrated that type IV allergic dermatitis induced by picryl chloride or oxazolone was suppressed in PAR-2 deficient mice.¹² Further, Schmidlin *et al.* reported that OVA-induced IgE production, airway eosinophilia and hyperreactivity to methacholine were impaired in PAR-2 KO mice.¹³ Using clinical biopsy specimens, up-regulation of PAR-2 expression in respiratory epithelium from patients with asthma has been observed.¹⁴ Together, these results clearly sug-

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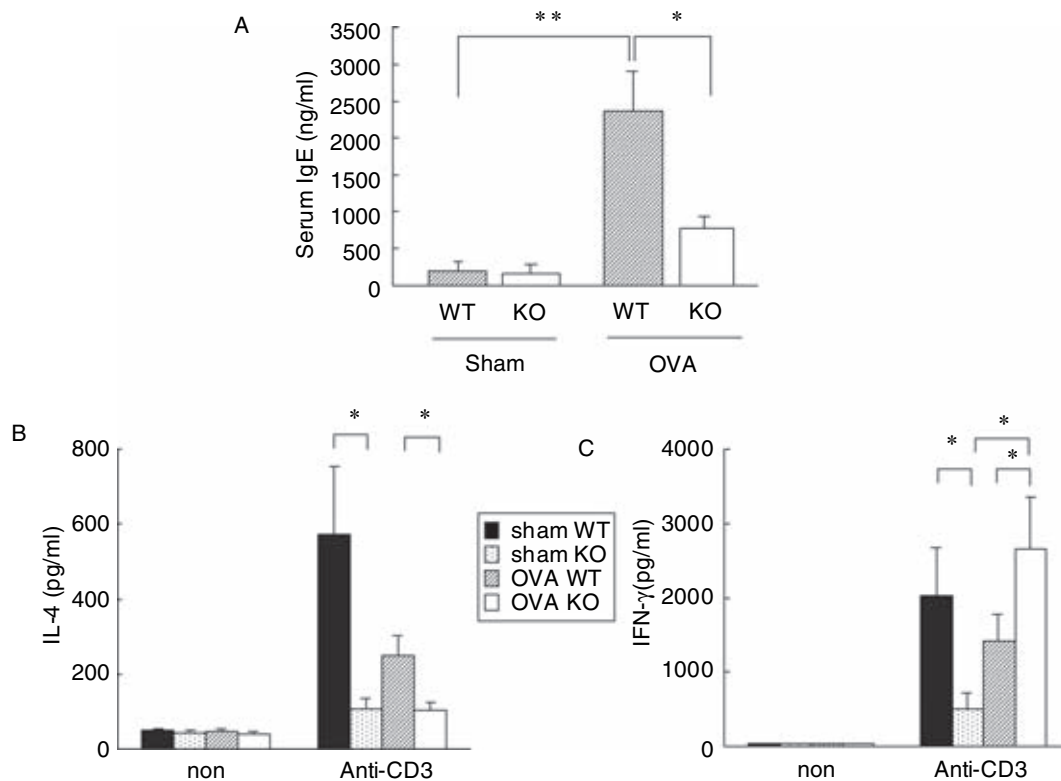


Fig. 1 OVA-induced serum IgE-production and anti-CD3-induced production of IL-4 and IFN- γ from splenic CD4⁺ T cells in WT and PAR-2 KO mice. Mice were sensitized and challenged with OVA (OVA) or sensitized with alum alone (Sham) as described in the Methods. Twenty-four hours after final challenge mice were euthanized, and sera and spleens were collected. Splenic CD4⁺ T cells were purified and then re-stimulated in pre-coated plates with 10 μ g/ml anti-CD3 mAb for 48 hours. The amount of IgE in serum, and cytokines in cell supernatants were measured by ELISA. Columns represent mean \pm SEM of 5–7 mice.

gest that PAR-2 plays a significant role in allergic airway inflammation.

Allergic asthma is a complex inflammatory disease associated with airway hyperresponsiveness (AHR), infiltration of eosinophils into the bronchial wall and lumen, mucus production in the airways and elevated serum IgE levels.^{15,16} CD4⁺ Th2 cells and their cytokine products, including IL-4, 5, 9 and 13, play critically important roles in the pathogenesis of asthma.¹⁷ Adoptive transfer of effector Th2 cells into naïve mice followed by exposure to inhaled antigen induces the pathophysiological features of asthma,¹⁸ demonstrating that these cells are fully capable of inducing the asthma phenotype.

In this study, we made use of PAR-2 knockout mice to examine the role of PAR-2 in cytokine production. In the model of OVA-induced airway inflammation, the cytokine pattern of T cells from PAR-2 KO and wild type (WT) mice were compared. Furthermore, cytokine secretion, gene expression in naïve T cells from PAR-2 KO and WT mice were analysed. To determine the difference of cellular signal, phosphoryla-

tion of JNKs in naïve CD4⁺ T cells was also investigated.

METHODS

GENERATION OF PAR-2-DEFICIENT MICE

Mouse PAR-2 genomic clones were obtained by PCR amplification. A targeting vector was constructed by subcloning the genomic DNA, a hygromycin selectable marker, and a lox P sequence into the ppNTT-neo vector. Ten mg of the targeting vector was introduced into RW4 ES cells by electroporation and stable clones were selected by culturing the cells for 10 to 12 days in the presence of 260 μ g/ml of G418. The clones were injected into C57 blastocytes. Germline transmission of the disrupted locus was confirmed by backcrossing the chimeric males to C57 females, and the homozygous knockout mice were obtained by crossing the chimeric males to 129SvJ females. Control mice (B6129 F2/J) were purchased from Jackson Laboratory (West Grove, PA, USA) according to their recommendation.¹⁹ S129SvJ background mice and their WT counterparts (Jackson Laboratory) were

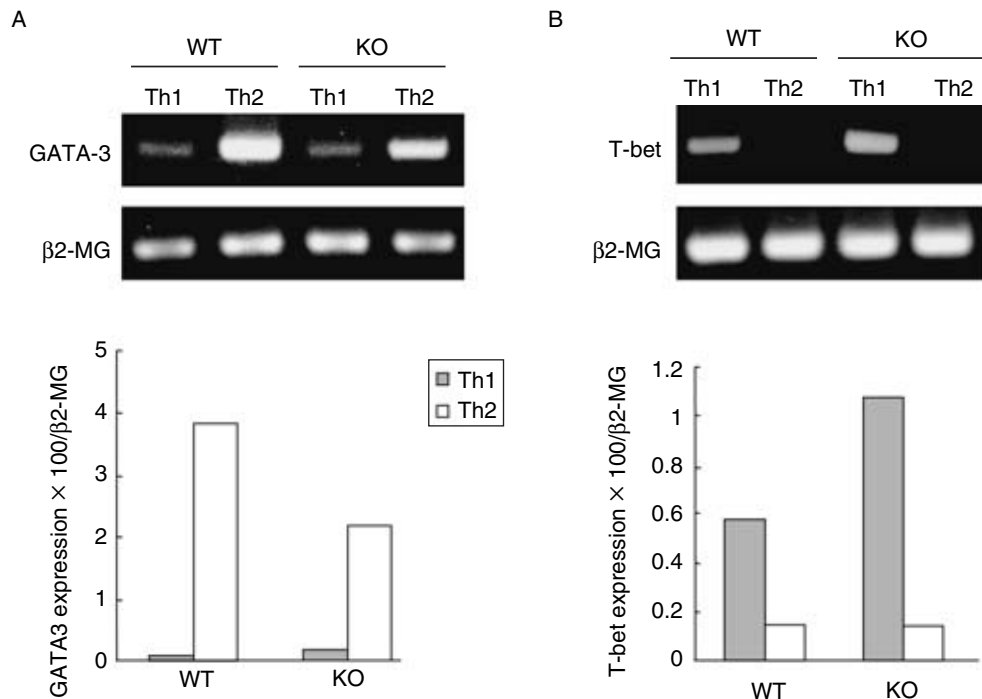


Fig. 2 Expression of mRNA for GATA-3 and T-bet after *in vitro* polarization of splenic CD4⁺ T cells toward Th1 or Th2. Purified splenic naive CD4⁺ T cells were cultured in the condition toward Th1 or Th2 (see Methods). After culture for 3 days, cells were lysed with Trizol, RNA was extracted and the expression levels of GATA-3 and T-bet were detected by electrophoresis or quantitatively measured by light cycler. Data shown represent one out of 2 independent experiments with identical results.

also used as described in previous studies.¹³

Animals were kept under standard conditions in a 12 h day/night rhythm with free access to food and water *ad libitum*. All animals received human care and the studies were approved by the internal ethic committee in accordance with the guidelines recommended by JALAS (Japanese Association of Laboratory Animal Science).

OVA-INDUCED IgE PRODUCTION

Mice were sensitized by intraperitoneal (i.p.) injections of 100 µg of ovalbumin (OVA, chicken egg, Grade V, Sigma-Aldrich, St. Louis, MO, USA) and 20 mg of alum (aluminium potassium sulfate dodecahydrate, Sigma-Aldrich) in 200 µl of saline on days 0 and 14. Mice in the sham group received i.p. injections of 20 mg of alum in 200 µl of saline. On days 14, 18 and 22, mice were challenged by intranasal (i.n.) administration of 100 µg of OVA in 40 µl of saline. Mice in the sham group received i.n. administration of 40 µl of saline only. Total IgE level in serum was measured by ELISA (Yamasa Co., Tokyo, Japan).

CYTOKINE PRODUCTION

CD4⁺ T cells were separated by using the mouse CD4 negative selection kit and MACS system according to

the manufacturer's instructions (Milteny, Bergisch Gladbach, Germany). Purified cells (purity >95%) from WT and PAR-2 KO mice were suspended in RPMI1640 containing 10% FBS and cultured at 2×10^5 cells/well in 96-well plates coated with anti-CD3 ε mAb (10 µg/ml, PharMingen, San Diego, CA, USA) for 48 hours at 37°C in an incubator. The amount of IL-2, IL-4, IL-13 and IFN-γ in the supernatants of the cultured cells was measured by ELISA (Genzyme Techne, Minneapolis, MN, USA).

POLARIZATION OF CD4⁺ T CELLS

Purified CD4⁺ cells were cultured under Th1-favouring conditions in the presence of 5 µg/ml anti-CD28 mAb (PharMingen), 50 IU/ml IL-2 (Pepro Tech, Inc., Rocky Hill, NJ, USA), 5 ng/ml IL-12 (Pepro Tech, Inc.) and 10 µg/ml neutralising anti-IL-4 mAb (PharMingen) or Th2-polarized conditions in the presence of 5 µg/ml anti-CD28 mAb, 50 IU/ml IL-2, 50 ng/ml IL-4 (PharMingen) and 10 µg/ml neutralising anti-IFN-γ mAb (PharMingen) as described in previous report.²⁰ After that, cells were restimulated in 12 well plates pre-coated with 10 µg/ml anti-CD3ε for 3 days. Total RNA from lysed cells was extracted and PCR was performed to detect mRNA for GATA-3 (Th2-specific transcription factor) and T-bet (Th1-

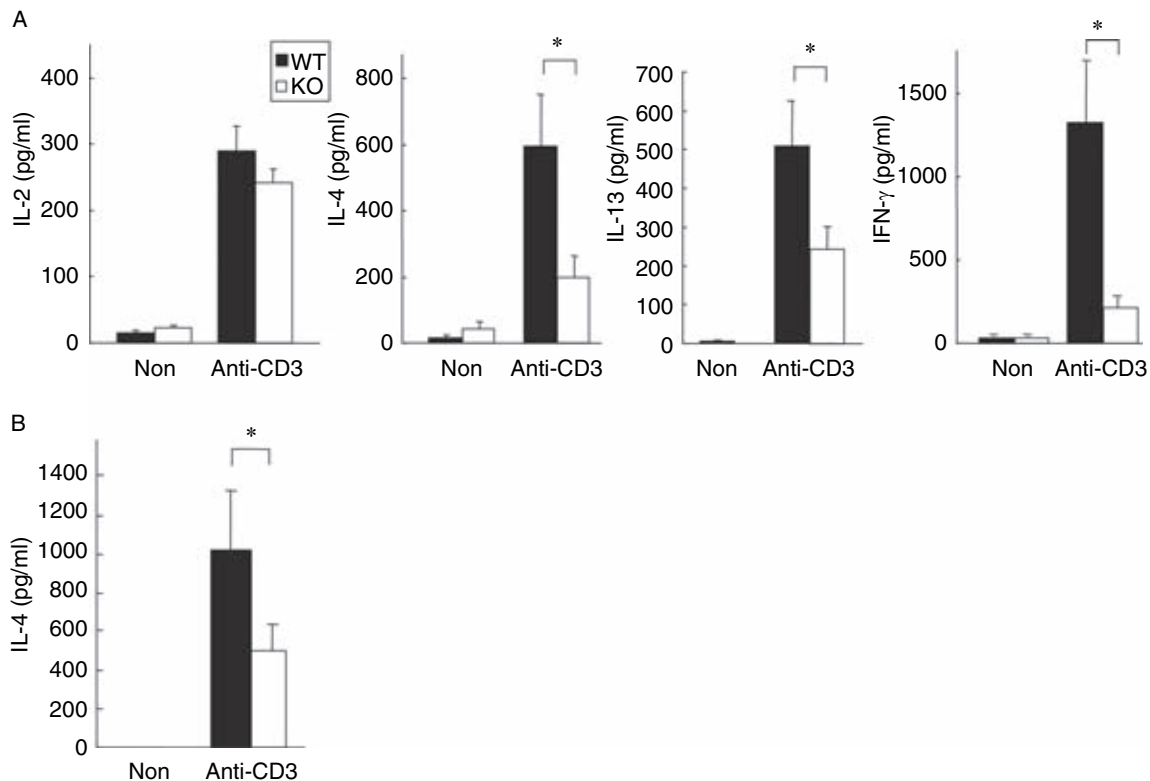


Fig. 3 Anti-CD3-induced cytokine production from purified splenic naive CD4⁺ T cells. Comparison of anti-CD3-induced production of IL-2, IL-4, IL-13 and IFN- γ from splenic CD4⁺ T cells between WT and KO of B6129 background mice (**A**). Anti-CD3-induced IL-4 production from Th2 polarized cells of S129 background WT and PAR-2 KO mice (**B**). Purified naive CD4⁺ T cells were cultured in 10 μ g/ml anti-CD3 pre-coated plates for 48 hours. The amount of cytokines were measured by ELISA. Data represent mean \pm SEM of 3–6 experiments. Significant difference between WT and PAR-2 KO T cells: * $P < 0.05$.

specific), respectively.

DETECTION OF mRNA EXPRESSION BY REAL-TIME PCR

Total RNA was isolated from CD4⁺ T cells after polarization into Th1 or Th2. The first strand cDNAs were synthesised with random hexamer primers and SuperscriptTM II RNase H- reverse transcriptase (Invitrogen, San Diego, CA, USA). The PCR product for GATA-3 and T-bet obtained in the dual PCR reaction was measured by SYBR green using LightCycler (Roche Diagnostics Co., Indianapolis, IN, USA). Detection temperature was 88°C for GATA-3, 86°C for T-bet and 80°C for β 2 microglobulin, and the annealing temperature was 65°C for all molecules. The following primers were used: mouse GATA-3, 5'-tcactctcgaggcagcatgaccac-3' and 5'-tcacacctccctgcctctgtgc-3'; mouse T-bet, 5'-ggttgagggtctctgggaagctga-3' and 5'-ggtgctggtccaccaagaccacat-3'; mouse β 2 microglobulin for a standard, 5'-gagaatgggaagccgaaca-3' and 5'-tatcagtctcagtgggggtg-3'.

WESTERN BLOT ANALYSIS

For the determination of CD3-mediated phosphorylation of JNKs, purified CD4⁺ T cells from WT and PAR-2 KO mice were stimulated with 10 μ g/ml anti-CD3 ϵ mAb or a combination of 20 ng/ml PMA and 1 μ M A23187 calcium ionophore for 15 minutes. Cells were lysed in lysis buffer (20 mM Tris, pH7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerol phosphate, 1 mM Na₃VO₄ and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)). The samples were separated by 10% SDS-polyacryl-amide gel electrophoresis. Gels were transferred to Immun Blot PVDF membrane and then incubated with blocking solution (5% skim milk in TBS, 0.1% Tween 20) for 60 minutes and then incubated with anti-phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling) and anti-SAPK/JNK (New England BioLabs, inc., MA, USA) diluted at 1/1000 with skim milk. Membranes were then washed and peroxidase-labeled anti-rabbit IgG (SantaCruz, CA, USA) diluted at 1/5000 with skim milk was incubated for 60 minutes. Membrane bound antibody was visualized using ECL detection

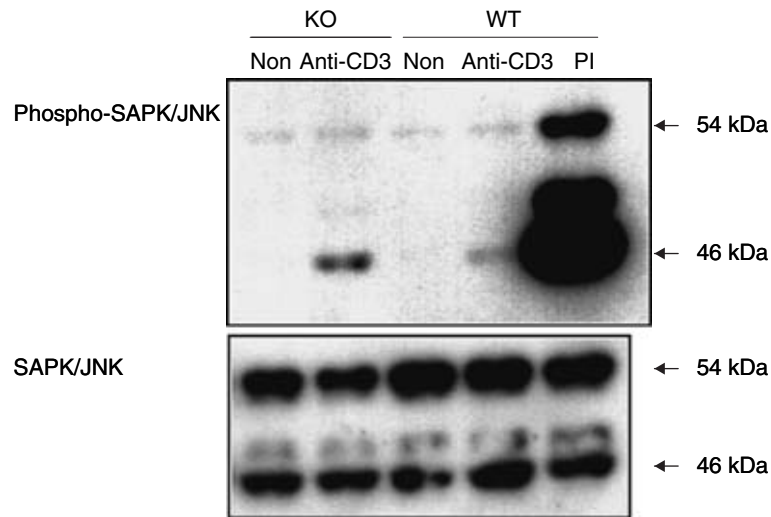


Fig. 4 Phosphorylation and expression of JNKs in CD4⁺ T cells. Purified CD4⁺ T cells were stimulated with 10 µg/ml anti-CD3 or a combination of 20 ng/ml PMA and 1 µM A23187 calcium ionophore for 15 minutes. Cells were lysed and separated by gel electrophoresis. Transferred membrane was incubated with anti-phospho-SAPK/JNK or anti-SAPK/JNK and then incubated with peroxidase-labeled anti-rabbit IgG. Membrane bound antibody was visualized by ECL detection reagent.

reagent (Amersham Biosciences, UK).

STATISTICAL ANALYSIS

Statistical significance of results comparing WT and KO mice, or sham treatment and OVA treatment was determined by Student's *t*-test for paired data. Differences were considered to be significant at the $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$). All data are expressed as the mean \pm SEM.

RESULTS

EXPRESSION OF PAR-2 mRNA

To verify the KO phenotype we compared PAR-2 mRNA expression levels in various tissues from WT and KO mice by RT-PCR using mouse-specific PAR-2 primers (5' -tctgatatcttctttccgaacg-3' and 5' -tacgagcagaaggttgctaggagc-3'). Spleen, thymus and liver from WT mice highly expressed PAR-2 mRNA whereas no signal at all was detectable in tissues from KO mice (data not shown).

REDUCTION OF IgE AND ANTI-CD3-TRIGGERED IL-4 PRODUCTION IN KO MICE AFTER OVA-STIMULATION

Reduced IgE production after OVA sensitization and challenge of mice lacking PAR-2 has been previously reported^{13,21} and was confirmed in our KO strain. As shown in Figure 1A, significantly lower levels of IgE in the serum of OVA-sensitized and OVA-challenged KO mice compared to WT counterparts were observed. To investigate whether reduced IgE was a

consequence of changes in cytokine production, we checked the Th1 and Th2 cytokine productivity by WT and KO mice. CD3-induced production of IL-4 and IFN- γ by spleen CD4⁺ T cells after sham or OVA-treatment was determined. Indeed, the release of IL-4 by CD4⁺ T cells from PAR-2 deficient mice was significantly reduced both after sham and OVA-treatment compared to WT mice (Fig. 1B). OVA stimulation tended to impair the CD3-induced IL-4 and IFN- γ production in WT cells compared to sham-treatment (Fig. 1B), suggesting that repeated stimulation by OVA might reduce cytokine production capabilities of splenocytes. Interestingly, the production of IFN- γ from CD4⁺ T cells was significantly higher in OVA-treated KO mice compared to OVA-treated WT mice or sham-treated KO mice (Fig. 1C).

SUPPRESSION OF GATA-3 AND UPREGULATION OF T-BET IN PAR-2 DEFICIENT CELLS

To further study the reduction of Th2 cytokine production after Th polarization in KO mice *in vitro*, purified naïve spleen CD4⁺ T cells were lead to a Th2 or Th1 phenotype. The expression levels of Th subtype-specific transcription factors GATA-3 (Th2) and T-bet (Th1) were determined. As shown in Figure 2A, high expression of GATA-3 mRNA was observed and clearly reduced expression levels were seen in KO cells in comparison to WT samples after polarization of cells to Th2. In contrast, when cells were polarized to Th1, clear expression of T-bet mRNA was observed and T-bet expression levels were up-regulated

in KO cells in comparison to WT cells (Fig. 2B).

CYTOKINE PROFILE OF NAÏVE CD4⁺ T CELLS

Since cytokine production not only differed between WT and PAR-2 deficient mice after OVA treatment but also after sham treatment (Figs. 1B, 1C), we next checked the cytokine profile of naïve CD4⁺ T cells. Since the background of our KO mice was a crossing between C57BL/6 and S129SvJ (see Methods for details), we first checked the cytokine production capabilities of those 3 mouse background strains. No significant differences were apparent following anti-CD3-induced production of IL-2, IL-4 and IFN- γ (data not shown). When PAR-2 KO mice were compared with the corresponding WT mice, a significant reduction of CD3-mediated IL-4, IL-13 and IFN- γ but not IL-2 release from naïve CD4⁺ T cells of KO mice was observed (Fig. 3A). This reduction in IL-4 protein secretion by naïve CD4⁺ T cells was further confirmed using S129SvJ pure background mice (data not shown). Moreover, a 35% reduction in IL-4 production was also noted after anti-CD3 re-stimulation of Th2 polarized cells from KO mice of the S129SvJ pure background when compared to WT cells (Fig. 3B).

EXPRESSION OF CD ANTIGENS AND MOLECULES RELATED TO T CELL FUNCTION IN WT AND KO CELLS

We then examined the protein expression of CD4 and CD8 and transcript expression patterns of several molecules related to T cell function to shed light on the potential influence of PAR-2 deficiency on the differentiation state and/or subtype development of naïve T cells. Surface protein expression of CD4 and CD8 both in spleen (CD4⁺CD8⁻; 31.9% in WT *vs* 32.3% in KO, CD4⁺CD8⁺; 15.8% in WT *vs* 16.3% in KO) and thymus (CD4⁺CD8⁺; 85.5% in WT *vs* 81.8% in KO, CD4⁺CD8⁻; 11.7% in WT *vs* 12.6% in KO, CD4⁺CD8⁺; 3.0% in WT *vs* 3.5% in KO) was unaffected by PAR-2 deficiency when checked by flow cytometry (mean of 2 experiments).

EXPRESSION AND PHOSPHORYLATION OF JNKs

The real-time RNA expression analysis by light cycler revealed that the expression level of JunB, JNK1 and JNK2 was not affected by PAR-2 deficiency ($n = 3$, data not shown). Moreover, protein expression levels of p46 JNK1 (Fig. 4), p54 JNK2 (Fig. 4) and JunB (data not shown) were not affected by PAR-2 deficiency. However, it is of interest that the phosphorylation of p46 JNK1 was clearly upregulated in KO cells compared to WT cells, whereas p54 JNK2 was not significantly affected by anti-CD3 stimulation (Fig. 4). By contrast, stimulation with PMA and A 23187 calcium ionophore significantly induced phosphorylation of both JNK1 and JNK2 (Fig. 4).

DISCUSSION

Involvement of PAR-2 in immune-related disorders has been suggested from *in vivo* studies using mice deficient for PAR-2 expression. It has been reported that eosinophil infiltration into the bronchoalveolar space, airway hyperreactivity to methacholine and serum IgE levels were suppressed in PAR-2 KO mice in an OVA-induced asthma model^{13,21,22} in which Th2 cells play a dominant role. On the other hand, so-called Th1-driven diseases, adjuvant arthritis²³ and type IV allergic dermatitis,¹² were also suppressed in PAR-2 deficient mice. The pathogenesis of these diseases involves multiple cell types and cytokines, yet a common feature in these animal models is immunization, implying a role of PAR-2 in affecting antigen-induced immune reactions.

In the present study, we confirmed the previous results^{13,21} of the reduced level of serum IgE in PAR-2 KO mice after OVA challenge. Concomitantly, we observed lower levels of IL-4 and higher levels of IFN- γ production from splenic CD4⁺ T cells from PAR-2 KO mice challenged with OVA. It was also acknowledged that both IL-4 and IFN- γ were lower in KO mice under sham treatment and only IFN- γ increased in response to OVA challenge. The diminished responsiveness to TCR stimulation with regard to production of Th1/Th2 effector cytokines seems to be intrinsic to T cells of mice lacking PAR-2. Specifically, CD4⁺ T cells isolated from the spleens of untreated mice, presumably naïve T-cells, readily showed reduced levels of IL-4, IL-13, and IFN- γ production in response to anti-CD3 stimulation. The decreased production of not only Th2 but also Th1 cytokines in the naïve CD4⁺ T cell population may provide an explanation for the suppressive effects of PAR-2 disruption reported for both asthma¹³ and arthritis²³ experimental animal models. Of particular interest in our results is the increased production of IFN- γ from splenic CD4⁺ T cells from PAR-2 KO mice challenged with OVA (Fig. 1C) and the increased expression of T-bet, a transcription factor regulating Th1 development, in splenic CD4⁺ T cells of PAR-2 KO mice after *in vitro* polarization towards Th1 (Fig. 2B). Regarding the upregulation of Th1, it should be noted that in the *in vitro* study at low concentrations of anti-CD3 Ab (less than 10 μ g/ml) the IFN- γ production by naïve PAR-2 KO T cells was downregulated, whereas at the highest concentration of anti-CD3 Ab (12.5 mg/ml) the IFN- γ production was at a similar level with wild cells (data not shown). Therefore, IFN- γ production in PAR-2 KO T cells might be changed by concentration, timing, kind and repetition of the stimulation, which might have induced conflicted output on Th1- and Th2-mediated experimental animal models.^{12,13,23}

Of note, the level of IL-2 production from the naïve T lymphocyte subset was not affected in PAR-2 KO mice. Indeed, there was no difference between PAR-2

KO and WT in *in vitro* T cell proliferation in response to TCR stimulation (data not shown). Thus, it is unlikely that the protective effects of PAR-2 disruption in the animal experimental models associated with immunological disorders are exerted by general immunosuppression.

The potential mechanisms that cause such an alteration of cytokine production from T cells are still matters of speculation. One theoretical possibility is that PAR-2 directly controls T cell function(s). In fact, the PAR-2 activating peptide, SLIGKV, has been reported to produce transient Ca²⁺ flux,²⁴ tyrosine phosphorylation of Vav1, ZAP-70 and SLP-70 in human Jurkat T cells²⁵ suggesting functionally active PAR-2 expression on this particular T cell line. Indeed, we detected a significant amount of PAR-2 transcript in mouse peripheral CD4⁺ T cells by RT-PCR analysis (data not shown). Nevertheless, mouse primary CD4⁺ T cells in the present study did not respond to any of the PAR-2 activating peptides tested (SLIGRL or t-LIGRLO) with calcium flux, Erk phosphorylation, or cytokine production (data not shown). Even when calcium flux and cytokine production were induced in combination with TCR activation no PAR-2 response was observed (data not shown). Although the possibility that PAR-2 might function at a certain stage of T cell development cannot be fully ruled out, so far there is no supporting evidence for a direct functional role of PAR-2 in mouse T lymphocytes.

In an attempt to identify a pathway affected by PAR-2 deficiency, we examined the expression of 32 genes including surface antigens, cytokine receptors, transcription factors and kinases in CD4⁺ T cells with microarray analysis (preliminary experiment, *n* = 1, data not shown). Of the genes associated with T cell function we have studied, none were influenced by PAR-2 deficiency. Notably, the expressions of transcripts and protein of JNK1, JNK2 and JunB were not affected, however, phosphorylation of JNK1 was clearly upregulated in KO cells in comparison to WT cells (Fig. 4). Activation but not expression of JNKs has been reported to be required for T cell activation,²⁶ therefore phosphorylation of JNK would affect T cell signaling and development. Dong *et al.*²⁷ demonstrated that JNK1 deficient T cells preferentially differentiated to Th2. Thus, upregulation of JNK1 might be associated with Th2 reduction and/or Th1 upregulation in KO cells. Phosphorylation of JNK2, whose deficiency leads to Th1 differentiation,²⁸ was not clearly affected in this study.

Recently, Fields *et al.* demonstrated that PAR-2 plays a role in the development of dendritic cells.²⁹ In their study, the differentiation of dendritic cells from precursor cells induced by GM-CSF and IL-4 *in vitro* was inhibited by serine protease inhibitors and by gene disruption of PAR-2. Conceivably, dendritic cell populations in PAR-2 KO mice might be different from those in the WT control mice. Although further

studies are required to fully comprehend whether particular dendritic cell subsets are affected by PAR-2 deficiency, it is intriguing to speculate that dendritic cell development is implicated in the alteration of T cell cytokine production in PAR-2 KO mice.

In conclusion, downregulation of IL-4 and upregulation of IFN- γ after antigen challenge in mice lacking PAR-2 were observed. Although the PAR-2 contribution to this phenomenon is not clear, PAR-2 may be involved in initiating the development of cytokine producing T cells at an early stage of development of the T cell lineage.

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