New role for the (pro)renin receptor in T cell development

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Key Points

- (Pro)renin receptor deletion in T cells drastically reduces the number of peripheral and thymic CD3+ T cells
- We identify multiple stages of thymocyte development which require (pro)renin receptor expression

Abstract

The (pro)renin receptor (PRR) was originally thought to be important for regulating blood pressure via the renin-angiotensin system. However, it is now emerging that PRR has instead a generic role in cellular development. Here, we have specifically deleted PRR from T cells. T cell-specific PRR-knockout mice had a significant decrease in thymic cellularity, corresponding with a 100-fold decrease in the number of CD4+ and CD8+ thymocytes, and a large increase in double negative (DN) precursors. Gene expression analysis on sorted DN3 thymocytes indicated that PRR-deficient thymocytes have perturbations in key cellular pathways essential at the DN3 stage, including transcription and translation. Further characterisation of DN T cell progenitors leads us to propose that PRR deletion affects thymocyte survival and development at multiple stages; from DN3 through to DN4, DP and single positive CD4 and CD8. Our study thus identifies a new role for PRR in T cell development.
Introduction

The (pro)renin receptor (PRR) was originally identified as a receptor for (pro)renin\(^1\). Its discovery led to an enormous amount of research in the cardiovascular field, due to the involvement of (pro)renin in promoting hypertension under conditions of renin-angiotensin system hyper-activation\(^2\). However, subsequent transgenic animal models failed to show a causal link between PRR and hypertension\(^2-5\). Instead, it has emerged that PRR is essential for cellular development and homeostasis. PRR is ubiquitously expressed and total knockout in mice is embryonic lethal\(^6\). Several PRR conditional knockout (cKO) models have been characterised, all with severe phenotypes: acute kidney injury with podocyte\(^7,8\), heart failure with cardiomyocyte\(^9\), and impaired eye and kidney development with retinal\(^10\) and uteric bud\(^11\) cKO.

Disturbances in many processes have been observed upon deletion of PRR\(^5,12\). These effects appear to be central to the molecular association of PRR with the vacuolar (V)-ATPase, where it is proposed that PRR regulates V-ATPase activity\(^13\). Much insight into PRR function has come from developmental biology studies, which showed the association of PRR with the V-ATPase is essential for canonical Wnt signalling\(^13\), an important developmental pathway. Further studies in \textit{Drosophila melanogaster} identified that knockdown of PRR perturbs trafficking of Wnt signalling receptors\(^14,15\). The role of Wnt signalling in T cell development has been controversial, with some studies showing a role\(^16\) and others not\(^17,18\). As PRR is highly expressed in lymphocytes\(^19\), we hypothesized that deletion of PRR from T cells would have implications for T cell development.

Methods

We bred \textit{ATP6AP2}\(^\text{flo}x/\text{y}\) females with male mice expressing Cre recombinase under the \textit{Lck} promoter (Fig S1). Male mice with PRR conditionally deleted from T cells (\textit{ATP6AP2}\(^\text{flo}x/\text{y}\);\textit{Lck}\(^\text{Cre}\)) are hereafter referred to as “cKO”. Full methods are available online.
**Results & Discussion**

The cellularity of peripheral lymphoid organs and blood were analysed by flow cytometry. In PRR cKO mice, a significant decrease in CD3$^+$ T cells was observed in all tissues, with no difference in the number of B, macrophage or dendritic cells (Fig 1A, Fig S2-3). A striking reduction in both CD4$^+$ and CD8$^+$ naïve T cells was evident, with no change in the number of peripheral TCR$\gamma\delta$ cells observed (Fig 1B). As PRR is found to be essential for homeostasis$^7$-$^11$, we asked if the remaining peripheral T cells had the PRR gene excised. We performed PCR that specifically amplified a segment of the PRR gene only after it was mutated by Cre recombination (Fig S4). Using this approach, we could detect the recombined gene (i.e. with the PRR gene excised) in thymic, but not peripheral CD90.2$^+$ T cells, which expressed similar levels of PRR protein as control T cells (Fig S4). Together, these results indicate that peripheral cKO T cells simply do not develop or survive. Instead, those few cells which do not recombine the PRR gene have an enormous selective advantage and reach the periphery.

The striking reduction in peripheral naïve T cells is consistent with a defect in thymic T cell development so we turned our focus to this organ. A severe atrophy of PRR cKO thymi was observed; evidenced by a reduction in weight and cellularity (Fig 1C). PRR cKO thymi had a 10-fold increase in the proportion of double negative (DN) thymocytes (Fig 1D). A significant increase in the proportion of intermediate single positive (ISP) and TCR$\gamma\delta$ cells was observed, whilst all other populations were decreased (Fig 1E). The number of DN cells was unchanged, whilst the number of ISP, double positive (DP), CD8 single positive (SP), CD4 SP and Tregs were reduced (Fig 1E). Histological analysis indicated the loss of SP T cells was associated with a reduction in the thymic medulla of cKO mice, but preserved cortico-medullary junction (Fig S5), consistent with other models of severe SP T cell loss$^{20}$. No change in the number of TCR$\gamma\delta$ cells was evident. This is unsurprising as the lineage commitment for TCR$\gamma\delta$ cells begins at the DN2 stage, prior to *Lck*-driven
Cre recombinase expression. Taken together, the almost 100-fold reduction in SP cells indicates that PRR cKO mice have drastically altered thymocyte development.

We next analysed early T cell developmental events (DN1-4). There was an increase in the proportion of DN3 cells in cKO mice, which was correlated with a decreased proportion of DN4 (Fig 2A). These proportional changes were not associated with differences in the number of DN3 or DN4 (Fig 2B). At the DN3 stage, cells commit to the T cell lineage and rearrange the T cell receptor (TCR) β-chain gene locus. PCR products corresponding to rearranged TCRβ locus were detected in both control and cKO cells, indicating that this process was not disturbed (Fig 2C). There was no difference in intracellular TCRβ levels (Fig 2D).

We next performed gene expression analysis of sorted DN3 cells (Fig 2E, Table S1A). In cKO, 615 and 518 genes were up- and down-regulated more than 1.5-fold. Of these, nine genes were significantly different, and encoded molecules important for processes including the cell cycle (Mcts1, Cetn2), mitochondria (Tomm7, Uqcrh) and vesicular trafficking (Ier3ip1, Rabac1). We next performed gene ontology (GO) analysis on the differentially expressed genes (Fig 2F, Table S1B). Several GO pathways were enriched in cKO DN3 cells, indicating disturbances in transcription, translation and the mitochondria. Collectively, these represent cellular activities essential for this developmental period, providing an explanation for why PRR deletion has such a profound effect on T cell development.

Comparing our findings to conditional deletion of the Wnt pathway in T cells, several similarities are evident: reduction in peripheral T cells and a change in the proportion of DN3:DN4. However, overall it is clear that PRR deletion induces a more profound phenotype. Wnt cKO led to an increase in the proportion of DN cells to 12%, and a partial block at the DN3-DN4 transition,
leading to an increased number of DN3 cells, and decreased DN4. In the periphery, there was a fourfold reduction in T cells, which did have the β-catenin deleted allele. In contrast, PRR cKO leads to an increase in the proportion of DN cells to ~50% of thymocytes, we detect no increase in the number of DN3 cells, and we find that DN4 numbers are decreased only in old mice (data not shown). Furthermore, we are unable to detect the deleted allele in peripheral T cells, indicating that PRR-deficient T cells do not develop/survive. To understand these results further, we sorted thymocytes and again performed PCR to specifically identify cKO cells. This revealed the presence of cKO cells beyond the DN3 stage, including DN4, DP and CD4 SP (Fig 2G). We analysed DP cells further and observed an impaired survival and, subsequently, rearrangement of the T-cell receptor α chain (Tcra) (Fig 2H-I). Thus, given the striking severity of our phenotype, that no one precursor thymocyte population accumulates, that we also observe defects in DP cells, we propose that deletion of PRR affects thymocyte survival and development from DN3 and beyond (Fig 2J).

Acknowledgements and disclosures

Thanks to Jana Czychi, Ilona Kamer, Sabine Schmidt and Dr. Hans-Peter Rahn for technical assistance. NHMRC supported KJB and MDW. DFG and DZH support DNM. SG, UM, MG, AM, KJB performed experiments. MK, RL, RD, OD gave advice. GN provided mice. DNM, MDW and KJB designed and wrote the study. The authors declare no conflict-of-interest.

References


Figure legends

**Figure 1. Abnormal thymic T cell development in PRR cKO mice**

A) Absolute numbers of T (all CD3^+), B, macrophages and dendritic cells from spleens of 7-8 week-old mice were determined by flow cytometry.

B) Absolute numbers of naïve (CD44^loCD62L^+) CD4^+ and CD8^+ T cells and γδT cells.

C) Morphology, tissue weight, cellularity, and number of CD3^+ cells of thymi from 6-week-old mice.

D) Thymocytes from 6-week-old control and cKO mice were analysed by flow cytometry. The gating strategy for CD4 and CD8 expression is shown.

E) From D: the percentage (top) and number (bottom) of double negative (DN), intermediate single positive (ISP), double positive (DP), CD4 single positive (SP), CD8 SP, γδ T cells and Tregs.

**Figure 2. PRR cKO impairs thymocyte survival and development from DN3 and beyond**

A) Representative flow cytometry of CD25 and CD44 expression in DN (CD4^−CD8^−) thymocytes from 39-day-old mice.

B) The frequency and actual number of DN3 and DN4 cells from A.

C) TCRβ gene rearrangement by PCR of sorted DN4 cells from control and cKO mice. DNA from bone marrow (BM) cells is shown as a non-rearranged control.

D) Expression of intracellular TCRβ in DN3 and DN4 thymocytes as from B.

E) Scatter plot showing the comparison of gene expression from microarray analysis of DN3 sorted cells from control and cKO mice. Significantly different genes (by 1-way ANOVA; q<0.05) are labelled (black circles). For clarity, the gene 5730437N04Rik is abbreviated as “5…Rik”, and the gene 1600029D21Rik is abbreviated as “1…Rik”. Inset shows PRR expression by real-time qPCR. N=3 biological replicates.

F) Gene ontology (GO) pathways which were significantly enriched in cKO DN3 cells (false-discovery rate (FDR) <0.5) are shown. Details of the genes in each pathway are listed in Table S1.
G) Representative PCR from sorted thymocytes from 39-day-old control and cKO mice to detect the unexcised PRR gene (“floxed”) and mutated/deleted gene in cKO (“excised”). Cre recombinase expression is also shown.

H) TCRα gene rearrangement was determined in sorted DP cells from control and cKO mice by quantitative PCR with primers specific for Vα8, Vα2 and Vα10 in conjunction with different Jα primers. The $p$ value shown for the effect of PRR deletion was calculated by two-way ANOVA. #\(p<0.01\) by Sidak’s multiple comparison test. N=3-4.

I) Thymocytes from control and cKO mice were cultured in vitro with IL-7 and the proportion of live cells was determined by flow cytometry at the desired time points. The $p$ value shown for the effect of PRR deletion was calculated by two-way ANOVA. **\(p<0.01\); ****\(p<0.0001\) by Sidak’s multiple comparison test. N=5-9.

J) Schematic of T cell development stages affected by PRR deletion.
Figure 1

Spleen

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Thymus

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Cells (x 10^7)

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Figure 2
New role for the (pro)renin receptor in T cell development

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