



# CD248 expression on mesenchymal stromal cells is required for post-natal and infection-dependent thymus remodelling and regeneration



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## ABSTRACT

**The role of mesenchymal stromal cells (MSCs) in regulating immune responses in the thymus is currently unclear. Here we report the existence and role of a MSC population in the thymus that expresses the pericyte and MSC marker CD248 (endosialin). We show using a CD248-deficient mouse model, that CD248 expression on these cells is required for full post-natal thymus development and regeneration post-Salmonella infection. In CD248<sup>-/-</sup> mice the thymus is hypocellular and regeneration is poorer, with significant loss of all thymocyte populations. This identifies the requirement of CD248 to maintain optimal thymic cellularity post-partum and infection.**

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## 1. Introduction

The thymus is a specialised microenvironment for the generation and selection of self-tolerant T-cells. Several studies have shown that mesenchymal-derived stromal cells (MSCs) play multiple roles in thymus development and function. While MSCs provide essential signals directly to the developing T-cell precursors [1], evidence suggests that MSCs can also influence thymic epithelial cell (TEC) populations. During embryonic thymus development, interactions between MSCs and TEC progenitors are required for the expansion of epithelial microenvironments, providing a finite number of niches that directly controls the efficacy of intrathymic T-cell production [2]. Without MSC support, an ineffective TEC network forms, naïve T-cell maturation is severely reduced and with it peripheral immunity [3,4]. While MSCs, including those of neural crest origin, clearly reside in the adult thymus, their function remains poorly characterised [5,6]. As well as dynamic changes in thymus volume during development, adult thymus commonly involutes during injurious threats such as infection, regenerating during resolution [7]. Therefore, understanding the biology of thymic MSCs and their role in the post-natal thymus is likely to help our understanding of thymic homeostasis and regeneration following involution.

CD248 (endosialin) is a member of a family of proteins involved in

tissue repair and remodelling [8,9]. Expression of CD248 is restricted to cells of mesenchymal origin [10]. In humans, CD248 is expressed on bone marrow MSCs [11], interstitial fibroblasts and perivascular cells [12–14] a niche where MSCs have been suggested to reside after birth [15]. We have demonstrated that murine CD248 is spatially and temporally expressed in lymph nodes where it regulates tissue expansion following immunisation [16]. In addition, high CD248 expression is observed during lymphoid tissue development. Expression decreases post-natally, but re-expression occurs during inflammation in adult tissue [17].

Given the requirement for MSCs in the formation of embryonic thymic microenvironments through their impact on TEC progenitors, and the dynamic expression pattern of CD248 on MSCs during development, we explored the possible role of CD248-expressing MSCs in adult thymic regeneration following involution. Here we show that CD248 is expressed on cells of the thymic capsule, and pericytes within the thymus. In addition, using the CD248<sup>-/-</sup> mouse model we show that CD248 expression is required for optimal post-natal thymic growth and regeneration following infection-dependent thymic atrophy.

## 2. Materials and methods

### 2.1. Mice, histology and confocal microscopy

Wild-type (WT) C57Bl/6 mice were obtained from Harlan UK Limited, Oxford, UK. eYFP C57Bl/6 mice from BMSU, Birmingham Uni-

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versity, UK. Generation of 129Sv CD248<sup>-/-</sup> mice has been previously described [18]. 129Sv CD248<sup>-/-</sup> mice were backcrossed onto C57Bl/6 mice >5 generations in-house generating CD248<sup>-/-</sup> mice used throughout. Frozen tissue sections were prepared, stained by indirect immunohistochemistry and analysed by confocal microscopy as described previously [16,17]. All experiments were performed in accordance with UK laws with approval of Local Ethics Committees.

## 2.2. Tissue digestion and flow cytometry

Thymuses were enzymatically digested as previously described [19]. Mature thymocytes were defined as CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> – double positive (DP) – CD45<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> – CD4<sup>+</sup> single positive (SP) – CD45<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> – CD8<sup>+</sup> SP – or CD45<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> – double negative (DN). DN subpopulations were defined as previously described [20].

## 2.3. Salmonella infections

Attenuated *Salmonella enterica* serovar Typhimurium SL3261 ( $5 \times 10^5$ ) were used to immunize adult (9- to 10-week-old) WT or CD248<sup>-/-</sup> mice via I.P. injection as previously described [21].

## 2.4. Determination of vessel length

Complete thymic lobes were stained with antibodies to CD31 and whole tissue analysed using a Leica DM6000. Four nonoverlapping areas of  $1 \times 1.7$  mm were chosen at random and using AF6000 Image Processing software, vessel length was measured. Data were tabulated and expressed as a percentage of the total number of vessels counted.

## 2.5. Statistics

Data are mean ( $\pm$  SEM) from three experiments, each with at least three replicates. *P*-values calculated by Student's non-paired *t*-test. \* *P* < 0.05, \*\* *P* < 0.01, \*\*\* *P* < 0.001.

## 3. Results

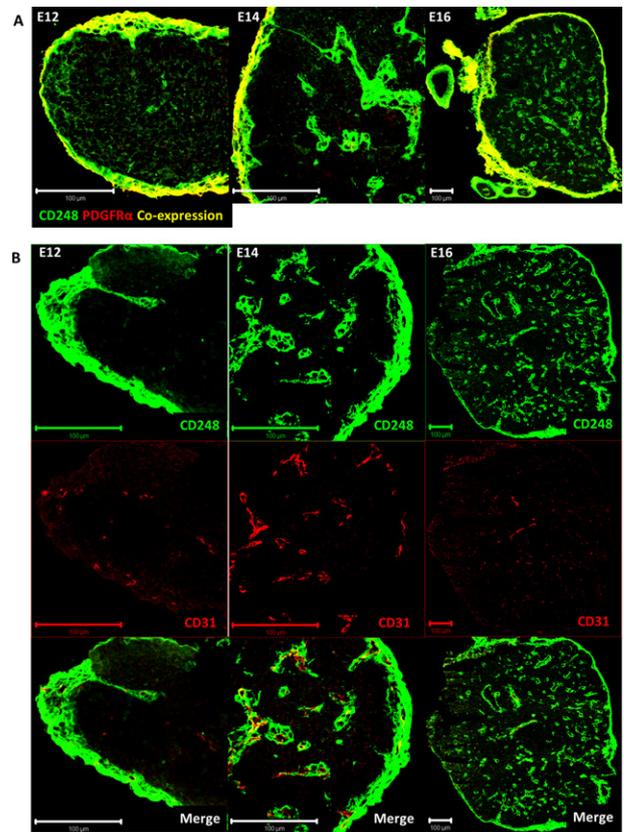
### 3.1. Location of thymic CD248

We have previously reported that thymic CD248 expression is downregulated post-natally, with little/no expression in the adult thymus [17]. During ontogeny CD248 expression is observed in the perithymic mesenchyme that forms the thymic capsule, co-expressed PDGFR $\alpha$  confirming their mesenchymal origin (Fig. 1A). In addition, CD248 is detected on perivascular cells which invaginate from the capsule to surround the CD31<sup>+</sup> vasculature (Fig. 1B).

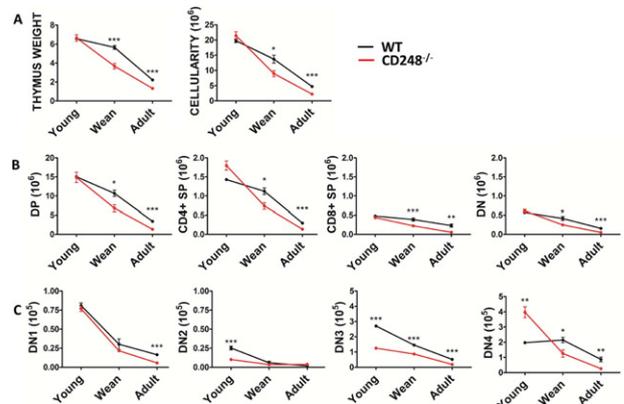
### 3.2. CD248 is required for optimal post-natal thymus growth

During post-natal development the thymus first undergoes rapid logarithmic expansion in size and T-cell output. Growth reaches homeostasis in adulthood, followed by age-related involution. CD248 expression decreases post-natally in the thymus but is re-expressed in lymphoid tissue during infection [16,17]. In addition, CD248 plays a role in regulating tumour expansion and metastasis [18], therefore we explored the role of CD248 during thymic development in WT and CD248<sup>-/-</sup> mice.

WT and CD248<sup>-/-</sup> thymuses from young (1–2 weeks old), weaning (3–4 weeks old) and adult (9–10 weeks old) mice were weighed and their cellularity enumerated. All data shown are expressed normalised to body weight, to compensate for any starting differences in animal size. Thymuses from CD248<sup>-/-</sup> mice were significantly smaller and contained significantly fewer thymocytes by the time



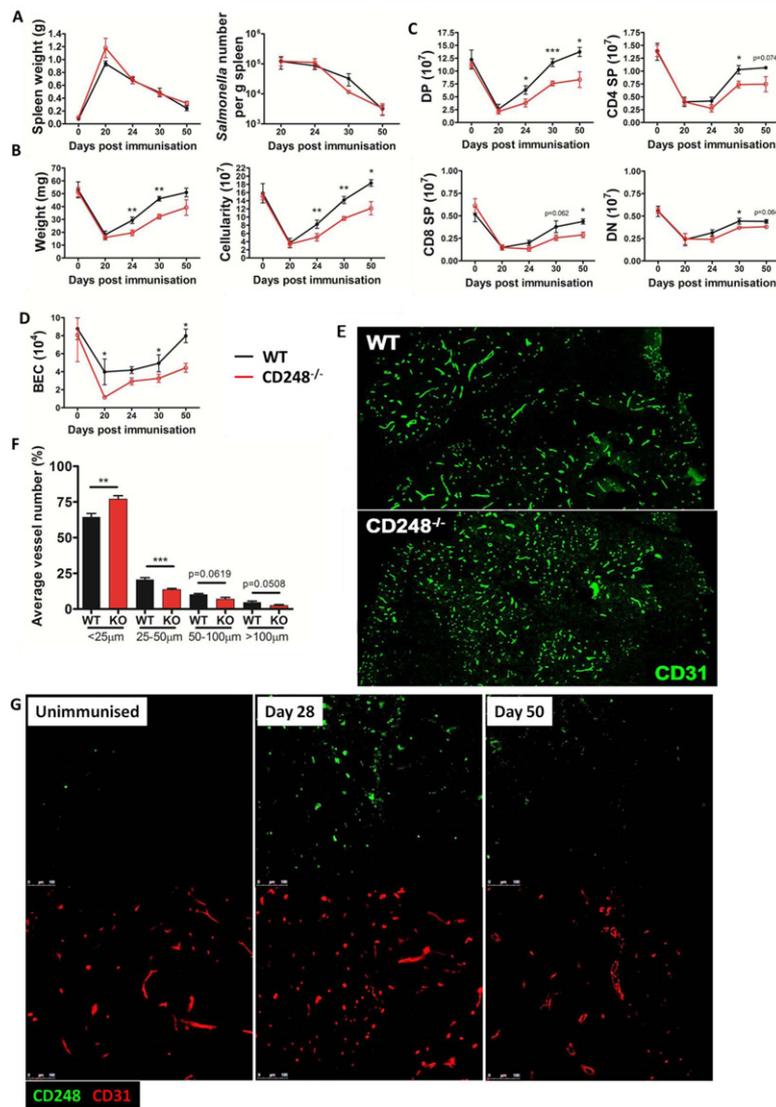
**Fig. 1.** Location of thymic CD248 expressing cells. Frozen sections of E12, 14 and 16 thymuses were co-stained with CD248 (green) and either PDGFR $\alpha$  (A) or CD31 (B) (red). Bar = 100  $\mu$ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** CD248 is required for efficient post-natal thymic development. Thymic weight and cellularity are reduced in young (1–2 weeks old), weaning (3–4 weeks old) and adult (9–10 weeks old) CD248<sup>-/-</sup> mice (A). Flow cytometry revealed a decrease in mature (B) and DN thymocyte populations (C) in CD248<sup>-/-</sup> mice.

of weaning compared to WT thymuses (Fig. 2A). This difference continued into adulthood, although reduced.

Flow cytometric analysis revealed these differences were predominantly due to a significant decrease in the number of double positive (DP) thymocytes, with significant decreases in total CD4<sup>+</sup> single positive (SP), total CD8<sup>+</sup> SP and double negative (DN) thymocytes also observed (Fig. 2B). Analysis of DN thymocyte subsets (DN1–4) revealed significant differences in all subsets at different times (Fig. 2C). It is interesting to note, that only DN3 thymocytes showed a consistent decrease in CD248<sup>-/-</sup> mice irrespective of age. These cells undergo rapid proliferation in the sub-capsular zone which has been suggested



**Fig. 3.** CD248 is required for infection-dependent thymic regeneration. *Salmonella* infection in CD248<sup>-/-</sup> mice, as assessed by splenomegaly and the number of bacteria per spleen, is similar to that of WT (A). Thymic weight and the number of bacteria per spleen are reduced during *Salmonella* infection-dependent regeneration in CD248<sup>-/-</sup> mice (B). Significant loss of thymocyte populations was observed in CD248<sup>-/-</sup> mice (C). Re-vascularisation of regenerating thymuses was dysregulated in CD248<sup>-/-</sup> mice as assessed by flow cytometry of BEC number (D), and endothelial vessel length in thymuses taken 30 days post-*Salmonella* infection (E and F). CD248 (green) is upregulated temporally during thymic regeneration (G). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to regulate thymic growth [22] and requires adhesion of the maturing thymocyte to extra-cellular matrix generated by MSCs [23].

### 3.3. CD248 is required for infection-dependent thymic regeneration

Adult thymus involutes during injurious threats such as infection, regenerating when that threat subsides [7]. Having observed that CD248 was required for optimal thymic growth during post-natal development, we reasoned the process of thymic regeneration following infection may also be linked to CD248 expression. Using a model of systemic *Salmonella* infection, thymic atrophy is observed, which by day 21 is accompanied by approximately 95% loss of DP thymocytes (Ross et al., submitted for publication [24]). This loss is followed by regeneration, with thymic cellularity and function re-established by 35 days post-infection.

Using this model, we assessed the role of CD248 during infection-dependent thymic regeneration using CD248<sup>-/-</sup> mice. The systemic response to *Salmonella* infection in CD248<sup>-/-</sup> mice is similar to that in WT mice, as assessed by splenic weight and *Salmonella* accumulation in the spleen (Fig. 3A). In contrast, thymic weight and cellularity

were both significantly reduced in CD248<sup>-/-</sup> mice during infection-dependent thymic regeneration (Fig. 3B). Assessment of CD4, CD8 thymocyte subsets during this regenerative-phase revealed a decrease in numbers of all subpopulations in CD248<sup>-/-</sup> mice (Fig. 3C).

It has already been reported that tumour growth and metastasis is reduced in CD248<sup>-/-</sup> mice due to dysregulation of neo-vascularisation; a significant increase in the number of small vessels was observed with a concomitant decrease in larger, more mature vessels in CD248<sup>-/-</sup> mice [18]. Therefore, we analysed the number of blood endothelial cells (BECs) during infection-dependent thymic regeneration in WT and CD248<sup>-/-</sup> mice to quantify vascularisation of the regenerating thymus. Thymuses were enzymatically digested and BECs enumerated following flow cytometric analysis (defined by CD45<sup>-</sup>EpCAM<sup>-</sup>gp38<sup>-</sup>CD31<sup>+</sup>). Prior to infection, the numbers of thymic BECs were not significantly different when comparing WT to CD248<sup>-/-</sup> mice. However, following thymic atrophy and during regeneration the numbers of BECs were significantly and consistently lower in thymuses lacking CD248 (Fig. 3D). Moreover, when vascular structures were stained with CD31 and their length measured 30 days

post-*Salmonella* infection, a significant increase in small vessels (<25  $\mu\text{m}$ ) was observed in CD248<sup>-/-</sup> thymuses (Fig. 3E and F). In addition, a significant decrease in 25–50  $\mu\text{m}$  vessels was observed in CD248<sup>-/-</sup> thymuses; a trend that was consistent in larger more mature vessels. Importantly, in WT thymuses perivascular CD248 expression increases during regeneration (Day 28) but is down-regulated again once infection is cleared (Day 50) (Fig. 3G).

#### 4. Discussion

Previously we identified a novel CD248<sup>+</sup> cell population required for efficient popliteal lymph node expansion following NP-CGG immunisation [16]. Here we show expression of CD248 on a population of thymic MSCs is involved in controlling thymic growth during post-natal development and infection-dependent thymic regeneration.

We have shown that in CD248<sup>-/-</sup> thymuses, vascularisation during infection-dependent regeneration is disrupted and that CD248 expressed on the perivascular cells plays an important role in controlling the size of the thymus and the number of mature thymocytes within the tissue. Interestingly, the reduced size of CD248<sup>-/-</sup> thymuses during regeneration correlates to growth of tumours transplanted into the abdomen of CD248<sup>-/-</sup> mice [18]. Therefore, these data suggest that CD248 regulates both infection and tumorigenic dependent re-vascularisation. It is interesting that in subcutaneous tumours no difference in tumour growth was observed between WT and CD248<sup>-/-</sup> [18] and during infection-dependent lymph node expansion the delayed growth of CD248<sup>-/-</sup> lymph nodes normalised over time [16]. This may reveal important site-specific differences in tissue re-vascularisation.

The finding that all thymocyte populations remain reduced in CD248<sup>-/-</sup> thymuses, even 50 days post-infection when WT thymuses have re-established function and cellularity, is an intriguing one. This raises the possibility that CD248 may be important when considering how thymic structure and function is re-established; not only in terms of therapies to boost immunity post-infection or treatment with chemotherapeutic agents (both of which can cause thymic involution), but also on age-related thymic atrophy. Therefore, CD248 may represent a novel molecule that links thymic vascularisation, function, and size.

Questions still remain, especially when considering possible mechanisms of how CD248 elicits its effect in thymic MSCs. We and others have shown that CD248 expression on MSCs confers a proliferative and migratory advantage [16,25,26], possibly via PDGFR signalling [27]. Having observed an increase in smaller, less mature vessels in CD248<sup>-/-</sup> thymuses during tissue regeneration, it is possible that optimal proliferation and migration of CD248<sup>+</sup> pericytes surrounding the developing vasculature promotes vessel stabilisation. Without sufficient pericyte support, vascularisation is stunted reducing the size of epithelial cell niches which permits optimal progenitor T-cell differentiation. One contributing factor that cannot be ruled out however, is possible differences in recruitment of Early Thymic Progenitor to the thymus of CD248<sup>-/-</sup> mice during development and regeneration, which may influence thymic size and cellularity.

Taken together these findings suggest CD248 plays a role in re-vascularising thymuses during infection-dependent regeneration, which may influence tissue size. Isolating these cells and examining their ability to alter thymic growth/regeneration and peripheral immunity when transferred into deficient environments will be of interest to investigate the contribution of MSCs in immune responses.

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