Impaired lymphoid organ development in mice lacking the heparan sulfate modifying enzyme glucuronyl C5-epimerase

Mark F. R. Vondenhoff*, Rogier M. Reijmers*, Jin-Ping Li, Ulf Lindahl, Steven T. Pals, Reina E. Mebius.

* These authors contributed equally

To be submitted
Chapter 6

Abstract

The development of lymphoid organs depends on the interaction between hematopoietic cells and mesenchymal stromal cells, and subsequently on the vascularization of the primordia. These processes are controlled by chemokines, cytokines and angiogenic factors that bind to heparan sulfate proteoglycans (HSPGs). Mice that are deficient for C5-epimerase (Glce−/−) cannot properly process HSPGs, which reduces their ability to bind molecules that are instructive for lymphoid tissue organogenesis. We hypothesized that this impaired binding capacity would negatively influence lymphoid tissue development. Here we show that impaired HSPG processing results in a reduced size of the fetal spleen (FS), while alterations in thymus morphology and lymph node development vary between the individual Glce−/− mice. The data indicate that correct HSPG processing facilitates lymphoid organ development, since deficiency does not lead to complete dissolution of these processes, although they occur less efficient.
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Introduction

Lymphoid organ development depends on the population of the lymphoid primordia by hematopoietic progenitor cells. Interactions between hematopoietic cells and mesenchymal stromal cells are necessary for the survival and further development of both these cell types and for the outgrowth of the primordium. While the source and nature of the inductive signals required for stromal cell differentiation in the primordia of the thymus and the spleen still need to be fully exposed, it has become clear that the signals for lymph node development emanate from lymphoid tissue inducer cells (LTI cells), which express lymphotoxin-α,β (LTα,β) and trigger lymphotoxin β receptor (LTβR) expressed on stromal cells. This results in the production of chemokines, such as CCL19, CCL21, and CXCL13, and adhesion molecules, necessary for the attraction and retention of additional LTI cells that will also contribute to the hematopoietic-mesenchymal cell interaction. Consequently, the primordium develops into a lymph node.

One of the key players in embryonic development are the heparan sulfate proteoglycans (HSPGs), as demonstrated by studies of model organisms and genetic defects in man. HSPGs are proteins with covalently attached polysaccharide heparan sulfate (HS) chains, they reside on the plasma membrane of all animal cells studied so far and are a major component of extracellular matrices. Native HS-chains consist of N-acetylated glucosamine (GlcNAc) and D-glucuronic acid (GlcA) repeating disaccharides, with a chain length ranging from as little as ten up to thousands of disaccharide units. For their function, the polysaccharide undergoes a series of modifications involving deacetylation, epimerization, and N- and O-sulfation. Ultimately, the HS-chains contain highly modified domains, which provide binding sites for many growth factors and nearly all cytokines. Binding of these proteins serves a variety of biological functions, such as cell adhesion and migration, angiogenesis, and tissue remodeling by the formation of gradients. An early modifier of heparan sulfate is C5-epimerase (Glce), which converts GlcA into L-iduronic acid (IdoA), thereby releasing the conformational constraints of the HS-chain, allowing better access of ligands for binding to specific regions of the polymer. Interestingly, mice deficient for Glce die neonatally with severe developmental abnormalities, including lung defects, skeletal malformations and renal agenesis.

Although many cytokines and all chemokines of the immune system contain HS binding capacities, the role of HS-modification in lymphoid organ development is still poorly investigated. Nevertheless, over the past decade, increasing evidence has demonstrated that HS-binding epitopes of chemokines, the structure of the HS-chain and chemokine oligomerization control the binding-specificity and functional outcome of chemokines. Taken these findings together, we hypothesized that lack of Glce will affect normal lymphoid organ development.

Here we show that Glce−/− mice suffer from severe abnormalities in lymphoid organ development. Spleen size is greatly reduced in all Glce−/− animals studied. In addition, most Glce−/− mice exhibit malformations in the vasculature of lymph node primordia, as well as changes in peripheral lymph node morphology.
Occasionally, inguinal lymph nodes were absent or ectopic lymph nodes were found. Furthermore, thymus development was affected ranging from thymi formed as a single lobe to dislocation of the right thymic lobe.

Materials and Methods

Mice
C57BL/6 mice carrying a mutant allele for glucuronyl C5-epimerase, described previously, were bred and maintained under specified pathogen free conditions at the animal facility of the Amsterdam Academical Medical Center (Amsterdam, The Netherlands). Mice were mated overnight, and the day of vaginal-plug detection was marked as E0.5. Pregnant females were sacrificed at E18.5 and embryos were harvested and examined either by immunofluorescence or FACS analysis. Offspring of the Glce+/- couples were genotyped by PCR, using primers as described before.

Macroscopic, histological, and immunofluorescence studies
To assess lymphoid organ development, embryos were autopsied at E18.5. Macroscopic images were taken on a Leica M2 FLIII using a Leica DFC 320 camera. Images were processed in Jasc Paint Shop Pro 7.

For immunofluorescence studies, 7 μm cryosections were fixed in dehydrated acetone for 2 min and air-dried for an additional 15 min. Endogenous avidin was blocked with an avidin-biotin block (Vector Laboratories, Burlingame, CA, USA). Sections were subsequently pre-incubated in PBS supplemented with 5% (v/v) mouse serum for 10 min. Incubation with primary Ab for 45 min was followed by a 30 min incubation with Fluor-Alexa-labeled conjugate (Invitrogen Life Technologies, Breda, The Netherlands) when needed. All incubations were carried out at room temperature. Before embedding in polyvinyl alcohol-based anti-fading mounting medium, sections were counter stained with Hoechst 33342 (Invitrogen Life Technologies, Breda, The Netherlands) for 10 min. Stainings were analyzed on a Leica TCS SP2 Confocal Laser Scanning Microscope (Leica Microsystems Nederland BV, Rijswijk, The Netherlands).

For histological studies, whole embryos were formalin-fixed and paraffin-embedded. Subsequently, 4 μm whole body serial tissue sections were cut and stained with hematoxylin (Merck, Darmstadt, Germany).

Antibodies
For immunofluorescence and flow cytometry, the following Abs were used: GK1.5 (anti-CD4), 53.67.2 (anti-CD8), MECA-367 (anti-mucosal addressin cell adhesion molecule-1 (MAdCAM-1), and MP33 (anti-CD45). All the Abs were affinity purified from hybridoma cell culture supernatants with protein G-Sepharose
Impaired lymphoid organ development in mice lacking the heparan sulfate modifying enzyme glucuronyl C5-epimerase (Pharmacia, Uppsala, Sweden) and biotinylated or labeled with Alexa-Fluor 488, Alexa-Fluor 546 or Alexa-Fluor 633 (Invitrogen Life Technologies, Breda, The Netherlands). 429 (anti-VCAM-1; BD Biosciences, Erembodegem, Belgium), 1A29 (anti-ICAM-1; BD Biosciences, Erembodegem, Belgium), anti-CD19, 145-2C11 (anti-CD3ε; eBioscience, San Diego, CA, USA), M5/114.15.2 (anti-I-A / I-E; Biolegend Europe BV, Uithoorn, The Netherlands), A7R34 (anti-Ill7r; eBioscience, San Diego, CA, USA), Avas12a1 (anti-VEGFR2; eBioscience, San Diego, CA, USA), MECA32 (pan-endothelial-cell marker; BD Biosciences, Erembodegem, Belgium), anti-Lyve-1 pAb (Millipore, Billerica, MA, USA), 11D4.1 (anti-vascular endothelial (VE)-cadherin; BD Biosciences, Erembodegem, Belgium), and anti-VEGFR1 pAb (anti-Flt1; Neomarkers Fremont, CA, USA) were used as biotinylated, phycoerythrin(PE)-conjugated, Alexa-647-conjugated or as unconjugated primary antibodies. 429, A7R34, Avas12a1, MECA32, anti-Lyve-1 pAb, 11D4.1 and anti-VEGFR1 pAb were visualized with Alexa-Fluor 488, Alexa-Fluor 546 or Alexa-Fluor 633 conjugated streptavidin, anti-rat IgG or anti-rabbit IgG. To assure specificity of the used Abs, conjugate-alone controls as well as control serum (rat or rabbit) as replacement of the primary incubation were used.

Flow cytometry
Expression of membrane proteins was analyzed by single, double, or triple stainings as described previously. Staining was measured on a FACS Calibur flow cytometer (Becton Dickinson, USA).

Results

Glce−/− mice display defects in thymus lobulation and positioning
Normal thymus development is initiated by the separation of the thymus primordia from the third pharyngeal pouch and their subsequent migration towards the anterior mediastinum to form a thymus consisting of two lobes, positioned at the midline. Neural crest cells that depend on BMP signaling for their existence are

<table>
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<th>Genotype</th>
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<th>Glce +/− (n = 14)</th>
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Table 1: Visual scoring of wild-type, Glce+/−, and Glce−/− mice for thymus position and lobulation. Unilobular and hypoplastic thymi and thymi of which a lobe was dislocated were determined aberant. Genotypes were confirmed by PCR.
Figure 1: Thymus development in Glce^-/- compared to wild-type mice. Macroscopic (A, B) and microscopic images (C-J) of E18.5 wild-type (A, C, E, G, I) and Glce^-/- (B, D, F, H, J) thymi consisting of only one thymic lobe. (C, D). Hematoxylin stained parafine sections of wild-type and Glce^-/- E18.5 fetusses show cortical (dark blue) and medullary areas in both wild-type (C, E) and Glce^-/- (D, F) thymi. (G, H, I, J) Immunofluorescent stainings of cryosections of wild-type and Glce^-/- thymi. (G, H) Double staining for CD45 and major histocompatibility class II (MHC II) I-A/I-E revealing CD45+ hematopoietic cells and MHC II I-A/I-E+CD45- thymic epithelial cells in both wild-type and Glce^-/- thymus (CD45 in red, MHC II I-A/I-E in green). The presence of these cells shows that the Glce^-/- thymus primordium is capable of attracting and retaining hematopoietic progenitors and that thymic epithelial cells necessary for thymocyte differentiation are present within the Glce^-/- thymus. (I, J) Triple stainings for CD4, CD8 and CD3 show that all thymocyte subpopulations found in wild-type E18.5 thymus primordia are also present in Glce^-/- littermates (CD4 in green, CD3 in red, CD8 in blue).
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Figure 1, continued.
involved in the migration of the two thymic lobes. In addition, thymus development requires the migration of neural crest cells into the thymus primordium, the colonization of the primordium by thymocyte precursors and the proliferation of precursors in later stages. While the colonization of the thymus by thymocyte precursors depends on the chemokines CCL21, CCL25, thymocyte proliferation requires CXCL12. Because BMP-signaling is modulated by HSPGs, and because HSPG binding properties of CCL21, CCL25 and CXCL12 were reported, we questioned whether defective C5 epimerization of HSPGs affects thymus development and function. Therefore, we compared the morphology and position, the histology, and the hematopoietic cell content of the thymi of wild-type (Glce+/+), heterozygous (Glce +/-) and knock-out (Glce-/-) littermates at E18.5 of gestation. Interestingly, we observed that whereas all wild-type (n = 7) and heterozygous (n = 14) mice showed a bilobular thymus positioned at the midline of the anterior mediastinum, 42% of the Glce-/- mice displayed distinct abnormalities in thymus lobulation and positioning (Table 1). Specifically, in two Glce-/- mice the thymi were unilobular and hypoplastic, but showed an orthotopic localization (Fig. 1A, B), while in 4 additional Glce-/- animals, the right thymic lobe was dislocated and positioned either anterior or lateral to the heart (data not shown). These results suggest that C5-epimerase modification of HSPGs is required for optimal control of the migration of the thymic primordia to their definitive position in the anterior mediastinum.

Despite the abnormalities in thymus positioning and lobulation, histological studies revealed no abnormalities in architecture of the thymi of the Glce-/- mice. Most importantly, a normal separation of cortical and medullary areas was present implying that thymocyte precursors had entered the tissue and assisted in organizing the tissue (Fig. 1C, D, E, F). Indeed, CD45+ hematopoietic cells were present in the thymus of both Glce+/+ and Glce-/- littermates. For the differentiation of T cell precursors into mature thymocytes, the interaction with major histocompatibility class II (MHC II) positive thymic epithelial cells is elementary. We therefore investigated whether MHC II I-A/I-E+CD45- thymic epithelial cells were present in Glce-/- mice. MHC II positive epithelial cells were indeed present in Glce-/- thymi (Fig. 1G, H), which indicated that thymocyte differentiation was not necessarily impaired in Glce-/- littermates despite the deficiency of C5-epimerase.

During the sequential steps in intrathymic T cell development, precursor cells proliferate and differentiate to acquire the accessory molecules CD4 and/or CD8 and the TCR-CD3 complex. At E18.5 in murine thymus development most thymocytes are double positive (DP) for CD4 and CD8 while negative or low for CD3, although low numbers of thymocytes that further differentiated into CD4+CD8+CD3hi and mature single positive (SP) CD4+CD8-CD3hi and CD4-CD8+CD3hi cells are already present. To reveal whether thymocyte precursors could develop into DP thymocytes in E18.5 Glce-/- littermates, we stained for CD4, CD8 and CD3. Indeed, we found CD4+CD8+CD3- thymocytes in Glce-/- thymi, and also mature SP CD4+CD8-CD3hi T cells (Fig. 1, J and Suppl. Fig 1). This shows that in Glce-/- thymus primordia, thymocytes are able to complete their differentiation program. Hence, C5 epimerase activity may be dispensable for the lymphopoietic function of the thymus.
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Figure 2: Fetal spleen development is altered in Glce<sup>-/-</sup> mice. (A) Cell counts of total fetal spleen (FS) cells from E18.5 littermates reveal a significant decrease in mean FS cell number of Glce<sup>-/-</sup> (-/-) FSs compared to wild-type (+/+) and Glce<sup>+/-</sup> (+/-) littermates. (B-D) Macroscopic images of representative (B) wild-type (+/+), (C) Glce<sup>+/-</sup> (+/-) and (D) Glce<sup>-/-</sup> (-/-) FSs. The reduced FS size observed in Glce<sup>-/-</sup> (-/-) corresponds with cell counts depicted in (A) showing decreased Glce<sup>-/-</sup> FS cell numbers. (E, F) Hematoxylin stained paraffin sections of wild-type and Glce<sup>-/-</sup> E18.5 fetuses containing FSs. The diameter of FSs is similar between Glce<sup>-/-</sup> and wild-type mice, indicating that especially FS length is affected by a deficiency in C5-epimerase. Spleens are indicated with arrowhead. * p < 0.05, ** p < 0.01, *** p < 0.001. (One-way ANOVA, Bartlett’s test, Tukey-Kramer test).
Glce\(^{-}\) mice show splenic hypoplasia but intact white pulp development

The red pulp of the murine fetal spleen (FS) has an important hematopoietic function. From day E14.5 onwards, a distinct white pulp anlage can be recognized by the presence of LTi cells, which are positioned adjacent to VCAM-1\(^{+}\)MAdCAM-1\(^{-}\)ICAM-1\(^{-}\) stromal cells, that surround major arterioles. To investigate the effect of C5-epimerase deficiency on spleen development, in particular on the formation of white pulp anlagen, we isolated FSs from day E18.5 murine fetuses. Macroscopic assessment revealed a severe size reduction of the FSs of all Glce\(^{-}\) mice.

<table>
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<th>Genotype</th>
<th>Glce (^{+/+}) (n = 16)</th>
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<td>Normal (%)</td>
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Table 2: Inguinal lymph node development of wild-type and Glce\(^{-}\) mice. Scores of inguinal lymph node development for individual wild-type, Glce\(^{+/+}\), and Glce\(^{-/-}\) littermates determined by macroscopy. When both inguinal lymph nodes could be distinguished, inguinal lymph node development was defined normal. Genotypes were confirmed by PCR.

(Fig. 2D) compared to both Glce\(^{+/+}\) (Fig. 2B) and Glce\(^{+/+}\) mice (Fig. 2C). Consistent with this size reduction, cell numbers in Glce\(^{-/-}\) FSs were also significantly reduced (Fig. 2A). The reduced FS size of Glce\(^{-/-}\) animals was primarily caused by a reduction in length, not by a reduction in FS diameter (Fig. 2E, F).

To explore the possible functional consequences of C5-epimerase deficiency on the development of the lymphoid compartment of the spleen, we studied the number and distribution of LTi and B cells, cells that are typically found during early splenic lymphoid development in the white pulp anlagen. FACS analysis demonstrated that the spleens of Glce\(^{-/-}\) mice contained normal percentages of B cells (CD19\(^{+}\)) while the relative number of LTi cells (CD4\(^{+}\)CD3\(^{-}\)CD45\(^{+}\)) were slightly but significantly increased (Fig. 3A, B) compared to Glce\(^{+/+}\) and Glce\(^{+/+}\) littermates. Immunofluorescent analysis revealed a similar clustered organization of LTi cells around arterioles (VEGFR1\(^{+}\)VEGFR2\(^{+}\)MECA32\(^{-}\)) in Glce\(^{-/-}\) and Glce\(^{+/+}\) littermates (Fig. 3C-H).

We have previously shown that adhesion molecules such as MAdCAM-1, VCAM-1 and ICAM-1 are expressed in the developing splenic white pulp. Although the expression of ICAM-1 was remarkable high at some locations in splenic white pulp of Glce\(^{-/-}\) mice (Fig. 3E, F), we did not observe a consistent change in the expression of these adhesion molecules in Glce\(^{-/-}\) mice. Hence, although C5 epimerase deficiency leads to a decrease in spleen size, C5-epimerase activity is not required for the first steps of splenic white pulp development.

Morphology of Glce\(^{-}\) lymph node anlagen

Lymph node development depends on the expression of chemokines such as CCL19, CCL21, and CXCL13. For several chemokines, such as MCP-1, CXCL1 and SDF-1, it was shown that their binding by HSPGs influences
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their signaling function and their availability to cells expressing the appropriate receptors [58 - 60]. To investigate the effect of altered binding properties of HSPGs on the formation of lymph node primordia, we performed macroscopic analysis of peripheral and mesenteric lymph node anlagen of Glce+/+, Glce+/-, and Glce-/- littermates. We found that C5-epimerase deficiency can abolish inguinal (Fig. 4A-E) but not mesenteric lymph node development (Fig. 4H, I). All Glce+/- mice (n = 13) displayed abnormalities in the development of blood vessel proximal to inguinal lymph nodes (Fig. 4B-E, Table 2), including abnormal position (Fig. 4E) and excessive branching of major blood vessels (Fig. 4B-E). However, only 69% of the Glce-/- mice lacked inguinal lymph node anlagen determined by macroscopy (Fig. 4B-D), while all Glce+/- animals that we investigated contained mesenteric lymph node anlagen similar to wild-type animals (Fig. 4F, G).

To study the development of lymph node anlagen in more depth, we analyzed sections of E16.5 and E18.5 wild-type and C5 epimerase fetuses by immunofluorescence. When sections were screened for the presence of lymph node anlagen by double staining for MAdCAM-1 and CD4, we found developmental differences in 80% of all Glce+/- animals studied (n = 5) when compared to their wild-type equivalents (n = 5). Differences observed were altered primordium size, ectopic primordium location, and abnormal primordium morphology visualized by MAdCAM-1+ stromal cells (data not shown).

Analysis of MAdCAM-1+Lyve-1+ lymphatic endothelial cells, VE-Cadherin+ vascular endothelial cells and MAdCAM-1+ lymph node stromal cells in peripheral lymph node anlagen, revealed that all non-hematopoietic cell types (Fig 4I) as well as CD4+IL7r+CD45+ LTi cells (data not shown) were present in Glce+/- lymph nodes. However, the distribution of MAdCAM-1+ stromal cells was only found in the direct vicinity of lymphatic endothelial cells that constituted the lymph node capsule and the overall number of MAdCAM-1+ stromal organizer cells seemed reduced. This capsule displayed an irregular morphology shown by its extensions into the primordium (Fig. 4H, I). The deficiency of C5 epimerase did not always result in aborted peripheral lymph node development however, since we could find lymph nodes at ectopic locations. These ectopic lymph nodes clearly contained features of normal lymph nodes, determined by the presence and organization of cellular subpopulations that are typically found in lymph node anlagen, such as CD4+IL7r+CD45+ LTi cells (Fig. 4J) co-localizing with VCAM-1+ICAM-1+ MAdCAM-1+ stromal cells (Fig. 4H and data not shown) and surrounded by Lyve-1+VEGFR2+ lymphatic endothelial cells (Fig. 45H, I). As in normal lymph nodes of wild-type mice, VEGFR2+MECA32+ (Fig. 4H) and VE-cadherin+VEGFR1+ vascular endothelial cells (Fig. 4H, I) were present within the anlagen. In summary, these results show that lymph node primordia of Glce+/- animals have the potency to attract and retain LTi cells that induce outgrowth of the primordium. The organization within these primordia however is affected by the C5 epimerase deficiency as shown by an altered morphology of most peripheral lymph node anlagen. In addition, the formation of inguinal lymph node anlagen can incidently be abolished.
Figure 3: Splenic white pulp anlagen can be found in Glce\textsuperscript{+} mice. (A, B) The percentage of CD4\textsuperscript{+}CD45\textsuperscript{+}CD3\textsuperscript{-} LTi (A) and B cells (B) of all CD45\textsuperscript{+} hematopoietic cells in Glce\textsuperscript{+}, Glce\textsuperscript{-}, and wild-type FSs were measured by FACS analysis. Each dot depicts the relative cell count for an individual mouse. (A) The mean relative LTi cell frequency of Glce\textsuperscript{-} FSs was increased when compared to Glce\textsuperscript{+} FSs. (B) There was no increase in mean relative B cell frequencies of Glce\textsuperscript{-} FSs opposed to Glce\textsuperscript{+} or wild-type FSs. (C, D) CD4\textsuperscript{+}Il7r\textsuperscript{+}CD45\textsuperscript{+} LTi cells were typically found in splenic white pulp anlagen in both wild-type and Glce\textsuperscript{-} FS (CD4 in green, Il7r in red, CD45 in blue). White pulp anlagen are indicated by white lines. (E, F) VCAM-1\textsuperscript{+} white pulp stromal cells (arrowhead) surrounding the ICAM-1\textsuperscript{+} central arteriole in both wild-type and Glce\textsuperscript{-} FSs (VCAM-1 in green, ICAM-1 in red, MAdCAM-1 in blue). (G, H) The central arteriole in white pulp anlagen of both wild-type and Glce\textsuperscript{-} expressed VEGFR1 and VEGFR2 but not MECA32 (arrowhead). In addition, in both wild-type and Glce\textsuperscript{-} splenic red pulp, small MECA32\textsuperscript{+}VEGFR2\textsuperscript{+} blood vessels were found (double arrowhead; VEGFR1 in green, MECA32 in red, VEGFR2 in blue). * p < 0.05, ** p < 0.01, *** p < 0.001. (One-way ANOVA, Bartlett's test, Tukey-Kramer test).
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Figure 3, continued.
The development of lymphoid tissue primordia depends on cellular cross talk and on the vascularization of the primordium. Signaling molecules that direct these processes, such as chemokines, cytokines and angiogenic factors, can bind to HSPGs. This binding to HSPGs is crucial for their instructive role during development. Here we show that a deficiency of C5 epimerase, an enzyme that is involved in the correct formation of HSPGs, results in abnormal lymphoid tissue development. All investigated \textit{Glce}$^{-/}$ animals suffered from a reduced spleen size. The individual differences among \textit{Glce}$^{-/}$ animals are profound for thymus morphology and lymph node development with regard to primordium size and location. However, the deficiency of C5-epimerase does not completely abolish the attraction and retention of hematopoietic progenitors, as we found the appropriate hematopoietic cells within lymphoid tissue primordia in all \textit{Glce}$^{-/}$ animals that were studied.

Although the deficiency of C5 epimerase does not prevent lymphorganogenesis per se, many abnormalities were found in developing spleen, thymus, and lymph nodes, indicating that the binding of signaling molecules to HSPGs facilitates in further increasing the efficiency of the cellular interactions required for lymphoid tissue development. Thus the ability of normally processed HSPGs to bind factors near their excretion point ensures the availability of these molecules at the desired location for lymphoid organ development, thereby facilitating morphogenesis.

Although Abramsson and coworkers reported previously that a deficiency in C5 epimerase only led to a mild vascular phenotype when compared to the severe phenotype of N-deacetylase/N-sulfotransferase-1 (NDST-1) knock-out mice \cite{61}. We show here that the relatively mild vascular phenotype of \textit{Glce}$^{-/}$ mice does in fact result in changed blood vessel development near lymphoid organ primordia. Also within lymph node primordia the vascular phenotype is abnormal. However, this irregularity does not block the formation of lymphoid tissue in the first phases of development, although ectopic development of lymph nodes was observed. Furthermore, in rare occasions when the position of major blood vessels was altered severely, lymphoid tissue development was completely abolished.

For thymus development the migration of neural crest cells into the developing primordial is indispensable \cite{62}. In this process neural crest cells, which rely on BMP for their existence, mediate thymic lobe migration \cite{63}, while the neural crest cells that migrate into the thymus primordium to form pericytes and vascular smooth muscle cells depend on PDGF-BB signaling during their development \cite{30, 31}. The latter cells are directly involved in the differentiation and proliferation of thymic epithelial cells. A deficiency in C5 epimerase might influence the contribution of neural crest cells to thymus development because of mildly affected BMP- and PDGF-BB signaling as reported \cite{61, 63}. This could explain the presence of hypoplastic or abnormal positioned thymic lobes in a number of \textit{Glce}$^{-/}$ mice.

Although we show here that altered blood vessel development within lymphoid tissue primordia does not completely block their development, blood vessels form
Impaired lymphoid organ development in mice lacking the heparan sulfate modifying enzyme glucuronyl C5-epimerase is an important component of lymphoid tissue primordia early in development. This is especially true for the FS, where a major part of the red pulp compartment is formed by blood vessels. It was therefore not surprising that our hypothesized effect of the C5 epimerase deficiency on lymphoid tissue development was indeed found in the spleen. We could not predict that this effect would be so severe with respect to FS size however, although a dramatic effect on FS development caused by an impaired blood vessel function was already reported previously. Nevertheless, both red pulp and white pulp anlagen could be distinguished in $\text{Glce}^{/-}$ spleens and both compartments contained all cellular subsets that were found in wild-type spleens. Because this was also true for thymus and lymph node anlagen, we conclude that the altered development of vessels is not decisive for the initial formation of the primordium.

To summarize our results, we show that impaired processing of HSPGs in $\text{Glce}^{/-}$ animals leads to a decreased spleen size in all animals. In addition, the organization and morphology of all lymphoid organ primordia varies among individual $\text{Glce}^{/-}$ mice. We conclude that this is a consequence of impaired binding of factors required for proper organogenesis, such as chemokines, cytokines and angiogenic factors to HSPGs at the location of the primordia that control the outgrowth and morphology of the lymphoid organ primordia.
Figure 4: Lymph node anlagen in wild-type and Glce−/− mice. Macroscopic (A-G) and microscopic images (H-L) of E18.5 wild-type (A, F, H) and Glce−/− (B-E, G, I-L) lymph node anlagen. (A) In wild-type animals, inguinal lymph node anlagen are typically located at the vascular junction junction (arrowhead) that are positioned at both sides of the body, between hypaxial musculature and skin. In Glce−/− mice, the morphology of these veins was highly variable between individual mice (B-F). Alterations observed were the parallel positioning of veins where normally a junction of veins is found (B); branching of numerous small blood vessels from larger veins (B, D); branching of few larger vessels from large veins (C); irregular shape of blood vessels (B, D, E). If present, the junction of large veins lacked inguinal lymph node anlagen in some animals (double arrowhead) (C, D), but could be distinguished in others (E) (arrowhead). In contrast to inguinal lymph node anlagen, mesenteric lymph node anlagen (G) were found in all Glce−/− animals, and were of similar morphology to wild-type mesenteric lymph node anlagen (F). (H, I) Microscopic images of wild-type and Glce−/− brachial lymph node anlagen, indicated by a white line. Both anlagen are positioned near a large VE-cadherin+ vein (arrowhead), and contain MAdCAM-1+VE-cadherin+ blood vessels and a capsule formed by Lyve-1+MAdCAM-1+ lymphatic endothelial cells. The Glce−/− BLN anlage contains an irregular shaped Lyve-1+ capsule and few MAdCAM-1+veolar cells (Lyve-1 in green, VE-cadherin in red, MAdCAM-1 in blue). (J, K, L) Glce−/− ectopic lymph node anlage indicated by white lines, proximal to fetal liver (FL; arrowhead). The ectopic lymph node anlage contains few LTi cells (CD4 in green, IL7r in red, CD45 in blue) (J). A small number of MAdCAM-1+veolar cells was located near lymphatic endothelial cells (Lyve-1 in green, VE-cadherin in red, MAdCAM-1 in blue) (K). VEGFR1−VEGFR2•MECA32α arteriole and a MECA32•VEGFR2• blood vessel proximal to the VEGFR2• capsule of the ectopic lymph node anlage (VEGFR1 in green, MECA32 in red, VEGFR2 in blue) (L).
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**Figure 4, continued.**
Reference list


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Chapter 6


Impaired lymphoid organ development in mice lacking the heparan sulfate modifying enzyme glucuronyl C5-epimerase

Supplementary Figure 1: Thymocyte development in Glce\(^{-/-}\) compared to wild-type mice. Confocal microscopic images of E18.5 wild-type (A,C,E,G) and Glce\(^{-/-}\) (B,D,F,H) thymi showing CD4, CD8 and CD3\(\varepsilon\) expression by murine thymocytes. Greyscale images (A,C,E, and B,D,F) depict single channels. Thymocytes expressing CD4 (A, B), CD3 (C,D), CD8 (E,F) are found in both wild-type and Glce\(^{-/-}\) thymi. Merge images (G, H) showing that CD4\(^+\)CD8\(^-\)CD3\(^{hi}\) thymocyte subsets are present in both wild-type and Glce\(^{-/-}\) thymus primordia. In addition, CD4\(^+\)CD8\(^-\)CD3\(^{hi}\) thymocytes (highlighted and enlarged) in Glce\(^{-/-}\) thymi indicate that thymocyte differentiation can continue until the terminal differentiation stage.