Thymus architecture in primary immunodeficiency and leukemogenesis

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Abstract

T lymphocyte development takes place in the thymus and relies on the constant seeding by bone marrow-derived progenitors. After arrival into the thymus, these progenitors commit to the T lymphocyte lineage and further differentiate to originate T lymphocytes. This is a rather complex process in which thymocytes migrate throughout the thymus, and according to the specific differentiation stage at which they are, occupy defined niches where they receive the necessary signals for further progression. Thymic epithelial cells (TEC) create these specialized niches in the thymus and are therefore essential for T lymphocyte development. Indeed, defects in the development of TEC impact T lymphocytes. The opposite is also true, and cell-autonomous defects in T lymphocyte development impact TEC development. This work has addressed the architecture of the thymus, i.e. its cellular structure in immunodeficiency and during leukemogenesis. Specifically, I analyzed histologically the thymus of immunodeficient mice and found that defects in T lymphocyte development led to impairments in TEC differentiation. Furthermore, using a model that involves thymus transplantations, we have analyzed the progression of a normal thymus during leukemogenesis. We observed that thymus structure is fully conserved while thymopoiesis is normal, but this changes with time, when pre-leukemic cells emerge and progress to T-cell acute lymphoblastic leukemia. Taken together, results show that defects in T lymphocyte development lead to impairments in TEC differentiation. Progression towards leukemia impacts both T cells and TEC. Further research is needed to clarify which of the described abnormalities precedes the other.

Keywords: T cell development, thymus development**,** thymic epithelial cells, primary immunodeficiencies, thymus autonomy, T-cell acute lymphoblastic leukemia.

Introduction

The thymus stroma, in particular the thymic epithelium, is responsible for providing the specific niches in which T lymphocytes differentiate. Likewise, thymocytes impact on the differentiation of T lymphocytes. Several examples of thymus crosstalk have been described in the literature. These include cases in which a defect in TEC leads to impaired T lymphocyte development, and the opposite, in which defects in T lymphocyte development impact on TEC structure and differentiation. Primary immunodeficiencies as well as thymus autonomy are examples in which there is a defect in T cell development that can lead to alterations in the thymic epithelium.

Primary immunodeficiency

Immunodeficiencies result from defects in the immune system. These can be divided in primary and secondary immunodeficiencies. Primary immunodeficiencies result from genetic defects leading to loss of immune cells or affecting their function, while in secondary immunodeficiencies these defects are due to external factors such as bacterial or viral infections, drugs that induce immunosupression, etc. Among the primary immunodeficiencies, severe combined immunodeficiency (SCID) is a disease in which more than one lineage of cells of the immune system is compromised. Patients with SCID require bone marrow or hematopoietic stem cell transplantation to survive [1]. These are rare diseases that manifest at young age. There are several forms, which can be divided into categories according to the cellular deficiency, i.e. absence of T, B and NK cells, and the genetic defect that causes the disease. The most common form is X-linked SCID (XSCID) which is $T^-B^+NK^-$ SCID and is characterized by few or even absent T and NK cells and results from defects in the γc gene. The common γc chain is part of the receptor for several cytokines, namely interleukin (IL-) 4, IL-7, IL-9, IL-15 and IL-21.

Thymus turnover versus thymus autonomy and leukemia

T cell development relies on the constant influx of bone marrow progenitors into the thymus and is characterized by high cellular turnover. Thymus transplantation experiments can be used to study a number of aspects related to T lymphocyte development, in particular those related to the dynamic nature of thymopoiesis. Transplants of wild-type thymi under the kidney capsule of wild-type mice demonstrated that the total replenishment of thymocytes takes around four weeks [2].

Classical experiments of transplantation of wild-type thymi into SCID or *Rag2*$^{-/-}$ mice, have shown that the wild-type thymus graft exports one single wave of T cells and then stops. This led to the generalized idea that thymocytes were short-lived cells and lacked the capacity to self-renew, which became a dogma of thymus and T cell biology. This dogma was challenged by work of two independent labs, which have shown that the thymus can maintain T lymphocyte production independently of bone marrow contribution under specific conditions [3][4]. Performing thymus transplants of wild-type newborn thymi into *Rag2*$^{-/-}\gamma c^{-/-}Kit^{W/Wv}$ recipients, which are devoid of progenitors capable of colonizing the thymus, T lymphocytes of donor origin continued to be produced and exported for unexpectedly long time. These results showed that, under specific conditions, thymocytes can become long-lived and acquire self-renewal capacity [3].

Since thymus autonomy developed from grafts into *Rag2*$^{-/-}\gamma c^{-/-}Kit^{W/Wv}$ hosts, new experiments were designed to identify the mutation that was permissive for thymus autonomy. Transplants of wild-type newborn thymi into *Rag2*$^{-/-}\gamma c^{-/-}$, *Rag2*$^{-/-}$*IL-7r*$^{-/-}$, $\gamma c^{-/-}$ and *IL-7r*$\alpha^{-/-}$ recipients revealed autonomous T-cell development, opposite for thymi grafted into *Rag2*$^{-/-}$ or *Kit*$^{W/Wv}$. These experiments revealed a key role for the presence of IL-7r in the T lymphocyte progenitors of donor origin [3][4][5]. Indeed, thymus autonomy leads to the development of T cells with a diverse TCR repertoire and capable of conferring immune protection to the host against infections [4][6].

Thymus autonomy leads to T-ALL in *Rag2*$^{-/-}\gamma c^{-/-}Kit^{W/Wv}$ *Rag2*$^{-/-}\gamma c^{-/-}$ and in $\gamma c^{-/-}$ recipients. These leukemias appear from 4 months after transplant onwards and have a CD8^{+} immature single positive/double positive-like phenotype both in spleen, bone marrow and in thymus grafts. T-ALLs that emerged from these experimental setups were similar to T-ALL in humans. In 80% of the cases, gain-of function mutations in *Notch1* were present, and *Tal1* and *Lmo2* were upregulated [5].

The main goals of the project were to address the thymus architecture in primary immunodeficiencies and in leukemogenesis. In the second goal, architecture was studied in the progression of thymus autonomy to T-ALL, in thymus autonomy from a *Rag2*$^{-/-}$ background and in multiple IL-7-unresponsive hosts.

Results

Thymus architecture in primary immunodeficiencies

During T lymphocyte development, thymocytes go through several stages of differentiation. To characterize the different cell populations in the thymus of wild type and immunodeficient mice, fluorescence-activated cell sorting (FACS) analysis was performed in thymocytes isolated from wild type, $\gamma c^{-/-}$, *IL-7r*$\alpha^{-/-}$, *IL-7*$^{-/-}$, *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*IL-7r*$\alpha^{-/-}$ and *Rag2*$^{-/-}\gamma c^{-/-}$ thymi. In a wild type thymus the cells proceed from the double negative stage (CD4^{-}CD8^{-}) to the double positive stage (CD4^{+}CD8^{+}) ending the differentiation process at the single positive stage (CD4^{+}CD8^{-} or CD4^{-}CD8^{+}). The majority of the cells are at the double positive stage (Fig. 1A). The same populations could be observed in $\gamma c^{-/-}$, *IL-7r*$\alpha^{-/-}$ and *IL-7*$^{-/-}$ mice (Fig. 1A) but thymi have a decrease in cell number (data not shown), which is due to lack of IL-7 signaling, essential for cell proliferation and survival especially at the double negative stage. As expected, all *Rag*-deficient thymi lack double positive and single positive thymocytes (Fig. 1A). Within the CD4^{-}CD8^{-} double negative (DN) cell populations, differential expression of cell-surface markers CD44 and CD25 can be used to identify four stages of T cell development, namely DN 1 (CD44^{+}CD25^{-}), DN 2 (CD44$^{+/lo}$CD25^{+}), DN 3 (CD44$^{-/lo}$CD25^{+}) and DN 4 (CD44^{-}CD25^{-}) cells. The majority of the cells are at the DN 3 stage. All immature stages from DN 1 to DN 4 are present in the thymus of $\gamma c^{-/-}$, *IL-7r*$\alpha^{-/-}$ and *IL-7*$^{-/-}$ mice. As expected the *Rag2*$^{-/-}$ thymus shows an accumulation of cells at the DN 3, consistent with the developmental block at this stage (Fig. 1B). This phenotype is exacerbated in the *Rag2*$^{-/-}$*IL-7r*$\alpha^{-/-}$ and *Rag2*$^{-/-}\gamma c^{-/-}$ thymi, resulting from combination of the developmental block at the DN 3 and the lack of IL-7 signaling (Fig. 1B).

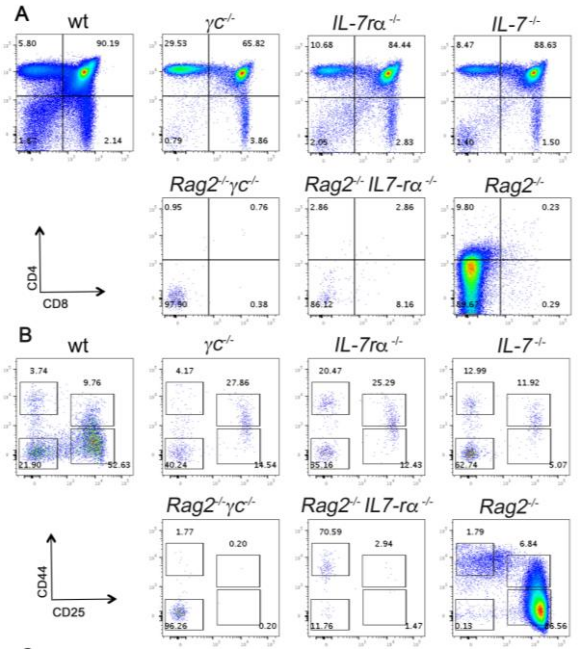

Figure 1- Differentiation stages of T cell development in immunodeficient mice. Thymocytes from the indicated genotypes were analyzed by FACS for CD3ε, CD4, CD8, CD25 and CD44. SYTOX blue was used to exclude dead cells. (A) Expression of CD4 and CD8 in live thymocytes. (B) Cells in A were further gated on CD4^{-}CD8^{-}CD3ε^{-} thymocytes and analyzed for the expression of CD44 and CD25. This experiment was performed once, using two animals from each genotype, 6 to 11 weeks of age.

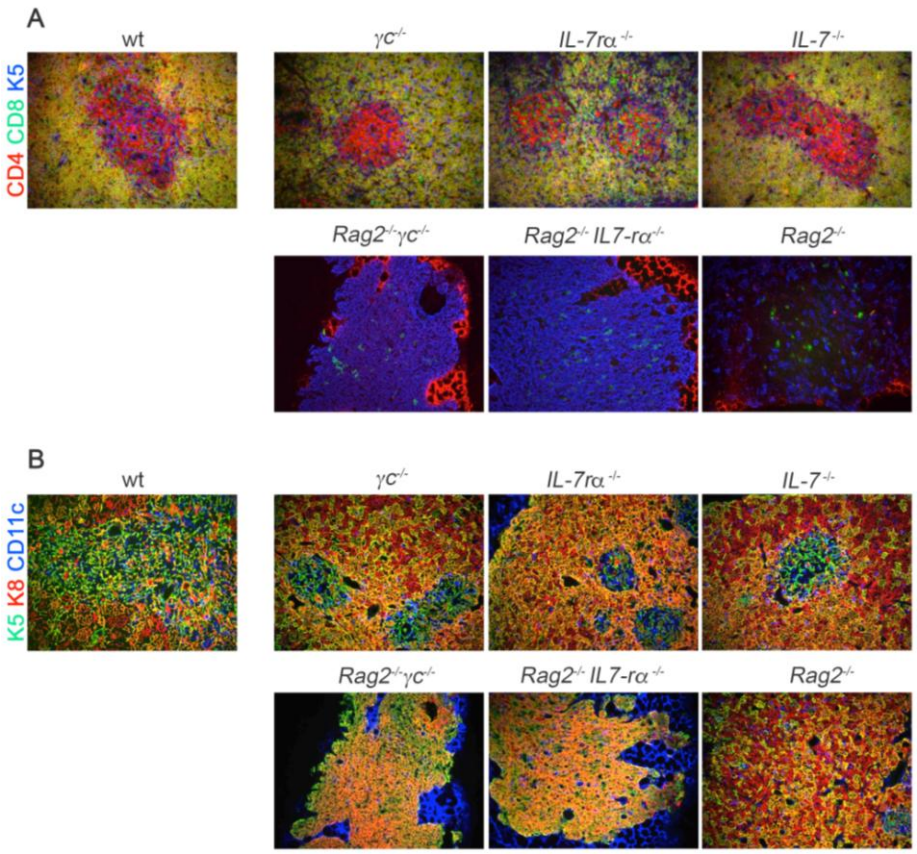

Figure 2- Thymus structure in mice with primary immunodeficiencies. Immunohistology of thymus sections of B6 wild type, $\gamma c^{-/-}$, *IL-7r*$\alpha^{-/-}$, *IL-7*$^{-/-}$, *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*IL-7r*$\alpha^{-/-}$, and *Rag2*$^{-/-}\gamma c^{-/-}$ mice, as depicted. (A) Staining for CD4 (red), CD8 (green) and K5 (blue). (B) Staining for K5 (green), K8 (red) and CD11c (blue). Images were acquired in Leica DMRA2 microscope with the software MetaMorph using a 20x magnification. This experiment was performed with 8 mice from 5 to 8 weeks of age.

Animals with the same genotypes were further analyzed by immunohistology to assess thymus structure. CD4 and CD8 identify the major populations of thymocytes and co-expression of the two markers defines $CD4^+CD8^+$ double positive thymocytes, which occupy the cortical region of the thymus in wild type, $\gamma c^{-/-}$, *IL-7r*$\alpha^{-/-}$ and *IL-7*$^{-/-}$ thymi. In all these thymi, $CD4^+$ and $CD8^+$ single positive thymocytes are located in the medulla, which can be further identified with basis on the presence of medullary thymic epithelial cells (mTEC), stained by cytokeratin 5 (K5) (Fig. 2A). The number of $CD4^+$ cells is bigger than the $CD8^+$ cells. As expected, all *Rag2* mutants lack double positive and single positive cells, consistent with the block in T cell development at the DN 3 stage. In these thymi, the thymus structure can only be identified with basis on the expression of K5 (and Dapi, not shown). The red staining outside the thymi corresponds to unspecific binding of the streptavidin to fat (Fig. 2A).

Beyond thymocytes, other cell populations were also analyzed, namely cortical thymic epithelial cells (cTECs), medullary thymic epithelial cells (mTECs) and dendritic cells (DCs). These cells were identified specifically by the expression of Keratin 8 (K8), Keratin 5 (K5) and CD11c, which stain mTECs, cTECs, and dendritric cells, respectively (Fig. 2B). A good separation between cortex and medulla could be observed, with basis on the differential expression of K5 and K8. Dendritic cells localized mainly in the medullary areas. Some TEC express both K5 and K8 (orange/yellow), which is thought to identify more immature cells, that are mostly restricted to the cortico-medullary junction [7]. The phenotype in $\gamma c^{-/-}$, *IL-7r*$\alpha^{-/-}$ and *IL-7*$^{-/-}$ thymi is identical to that of the wild type control, with a clear separation between cortex and medulla and the dendritic cells mainly localized in the medulla. However, these thymi had an increase in the proportion of TEC that co-expressed K5 and K8 in the cortical region. In all cases of *Rag2* deficiency, there were no medullary areas. Nevertheless, *Rag2*$^{-/-}$ thymi have TEC that are clearly positive for K5 but not K8, i.e. TEC that have the phenotype of mTEC (Fig. 2B). The thymus of *Rag2*$^{-/-}$*IL-7r*$^{-/-}$ and *Rag2*$^{-/-}\gamma c^{-/-}$ mice had a more severe phenotype, with a more compacted cellular structure than the *Rag2*$^{-/-}$, and with virtually all TEC co-expressing both K5 and K8 and presence of cysts. Also no dendritic cells could be identified in these sections (Fig. 2B).

Thymus architecture: from thymus autonomy to T-ALL

Thymus autonomy is the capacity of the thymus to maintain its function of generating T lymphocytes independently of bone marrow contribution. It is switched on in thymi that are deprived of competent progenitors and can be studied in a setting of thymus transplantation. If prolonged, thymus autonomy is permissive to the development of T-ALL. Thymus transplants were performed in the lab using newborn wild type donors transplanted into *Rag2*$^{-/-}\gamma c^{-/-}$ adult recipients. This experimental condition was always compared with wild type thymi transplanted into wild type recipients and wild type thymi that were not transplanted. In all cases, the origin of the cells, i.e. whether they were

of donor or host origin could be determined with basis on the differential expression of CD45.1 and CD45.2. To analyze the structure of the thymus during thymus autonomy, and determine whether it changes during the course of development of T-ALL, grafts were analyzed at several timepoints after transplantation, namely 28 days after transplant, an early stage of thymus autonomy, 9 weeks after transplant, a later stage of autonomy/pre-leukemic stage and a final timepoint, when T-ALL has already developed.

The earliest timepoint analyzed was day zero, corresponding to thymi from newborn mice, which were not transplanted. These were compared to adult thymi. Both newborn and adult thymi had a similar distribution of thymocytes, with double positive thymocytes present in the cortex and single positive cells localized in the medulla. Furthermore, medullary areas can be distinguished from cortical areas, with staining for K5 in the medulla and for K8 in the cortex and with dendritic cells predominantly present in the medullas (images not shown).

Day 28 after transplant

28 days after transplant corresponds to a timepoint in which thymus turnover has mostly taken place in a wild type scenario. However, if a wild type thymus was grafted into a *Rag2*$^{-/-}\gamma c^{-/-}$ recipient, we consider that 28 days after transplant corresponds to an early stage of thymus autonomy. Wild type thymi that were transplanted into wild type recipients are identical to thymi that were not transplanted. $CD4^+CD8^+$ double positive cells localized in the cortex, and $CD4^+$ and $CD8^+$ single positive cells are in the medullas, further identified by K5-positive mTECs. During autonomy, this structure is kept and the only difference is an increase in the proportion of $CD8^+$ single positive thymocytes in the medulla (Fig. 3A), consistent with our previous work, by FACS [3]. In addition, TEC staining and the localization of dendritic cells is identical in all tested conditions (Fig. 3C). The only difference that could be detected in thymus autonomy was that medullary areas were larger than in control conditions in which thymus turnover took place (images not shown).

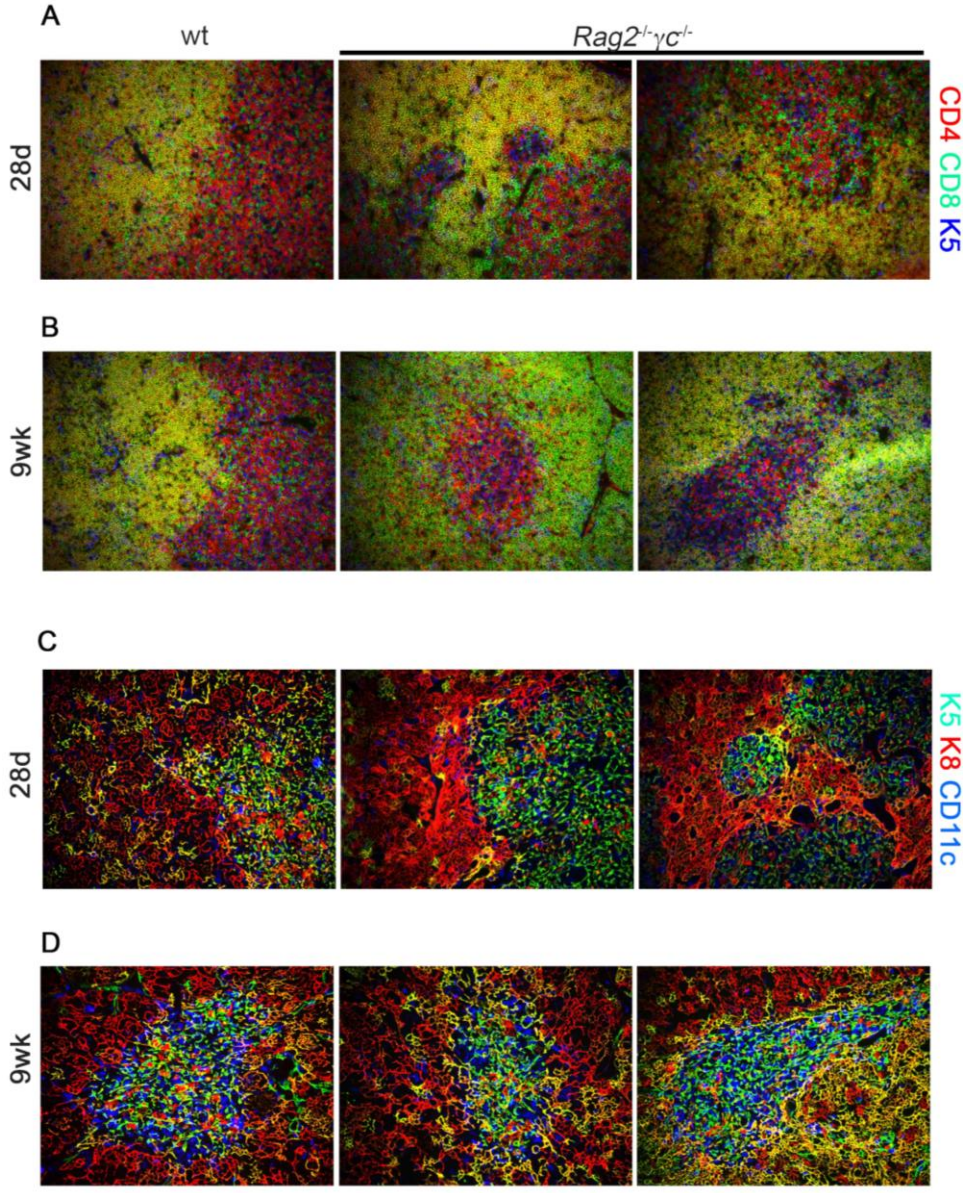

Figure 3- Thymus structure in thymus autonomy 28 days and nine weeks after transplant. Wild type newborn thymi were grafted under the kidney capsule of adult recipients (wild type or *Rag2*$^{-/-}\gamma c^{-/-}$, as indicated) and analyzed 28 days and 9 weeks later by immunohistology for the indicated markers, as depicted. Wild type B6 thymi grafted into wild type recipients were used as staining control. (A, B) Staining for CD4 (red), CD8 (green) and K5 (blue), as depicted. (C,D) Staining for K5 (green), K8 (red) and CD11c (blue), as depicted. Images were acquired in Leica DMRA2 microscope with the software MetaMorph using a 20x magnification. This experiment was performed with 23 mice.

Week 9 after transplant

Wild type thymi that were transplanted into wild type recipients and wild type non-transplanted thymi have the same phenotype. The cortical areas harbor $CD4^+CD8^+$ double positive cells, while the presence of $CD4^+$ and $CD8^+$ single positive cells is almost restricted to the medullas, identified by the cells that express K5. The majority of the grafts are broadly normal, characterized by having a similar phenotype to a wild type case (Fig. 3B). A second phenotype was also identified, corresponding to exhausted thymi, in which thymopoiesis was no longer supported. In the latter case no more $CD4^+CD8^+$ double positive thymocytes were found, and large areas of keratin staining only were visible. In those thymi, circulating $CD4^+$ or $CD8^+$ single positive cells were present in areas resembling former medullas (images not shown).

Analysis of TEC and dendritic cells also had some variation between thymi in autonomy. Some were identical to the control conditions, while others had some major changes. Even grafts in which T lymphocyte development appeared normal (Fig. 3B) could have changes in the epithelium, i.e. medulla and cortex could be easily identified but many cTECs that co-expressed K5 and K8, suggestive that the loss of cellularity in thymocytes occurring in autonomy particularly impacts on the cortical epithelium (Fig. 3D). The most extreme cases, corresponding to exhausted thymi correspond to a minority. These maintained the separation between cortex and medulla but the proportion of cells co-expressing K5 and K8 was increased in the cortex (images not shown). Dendritic cells maintain their normal localization in the medullas (Fig. 3D).

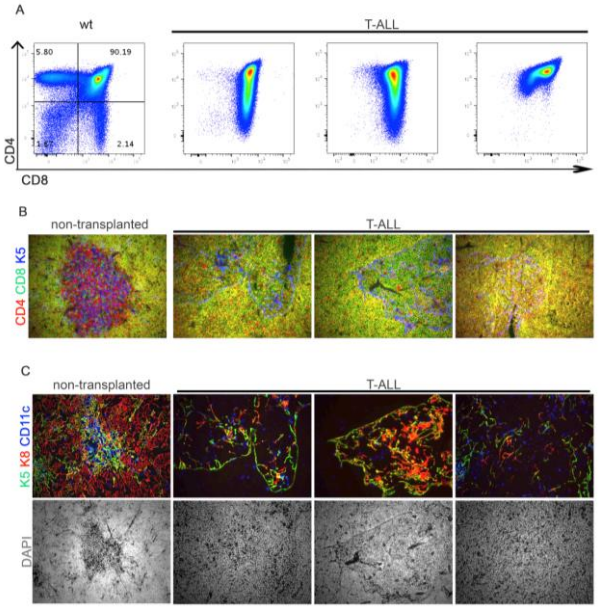

Figure 4- T-ALL is characterized by organ destruction with loss of the epithelium. (A) Thymocytes isolated from a wild type B6 adult and the thymus grafts in three T-ALL samples were analyzed by flow cytometry for the expression of CD4 and CD8. Thymocytes were stained with anti-CD3ε, anti-CD4, anti-CD8, and with SYTOX blue to exclude the dead cells. (B, C) Immunohistology in thymus sections of wild type adult thymus and grafts of T-ALL. Wild type B6 thymus was used as staining control. (B) Immunohistology of the thymus sections with staining for CD4 (red), CD8 (green) and K5 (blue). (C) Immunohistology of the thymus sections with staining for K5 (green), K8 (red) and CD11c (blue). Staining for Dapi is in grey, staining the nuclei of the cells. Images were acquired in Leica DMRA2 microscope with the software MetaMorph using a 20x magnification.

T-cell Acute Lymphoblastic Leukemia

T-ALL samples were analyzed by flow cytometry and I reanalyzed the files of the same samples posteriorly analyzed by immunohistology. Leukemic cells present an immature phenotype, expressing both CD4 and CD8, resembling the expression profile of thymocytes in a normal thymus but never found outside the thymus of a healthy mouse. The extent of CD4 or CD8 intensity differed from sample to sample, i.e. two of the T-ALL samples were $CD4^+CD8^+$ double positive and $CD8^+$ immature single positive, while in the other the majority of the cells were $CD4^+CD8^+$ double positive (Fig. 4A). This fits the staining observed by immunohistology, in which grafts showed varying intensities of the staining for CD4 and CD8 between samples (Fig. 4B). No normal structure was found, and the segregation between medulla and cortex was lost. Instead, an apparent organ destruction took place and epithelial areas were largely absent. The dendritic cells are still present but are scattered through all the tissue (Fig. 4C).

Comparing the different timepoints of study, covering from day zero until 28 days after transplant, the distribution of T cells, TECs and dendritic cells was kept normal but the medullary areas became larger. From 28 days to 9 weeks after transplant, thymus architecture was maintained in terms of T cell development but some cTECs started to co-express $K5^+K8^+$. From 9 weeks on, grafts with autonomy present the same phenotype as in 28 days after transplant in terms of T cell distribution, but there is an enrichment in cTECs that are $K5^+K8^+$. In T-ALL there was tissue destruction with leukemic cells occupying the entire organ and TEC largely absent (Fig. 5A, B).

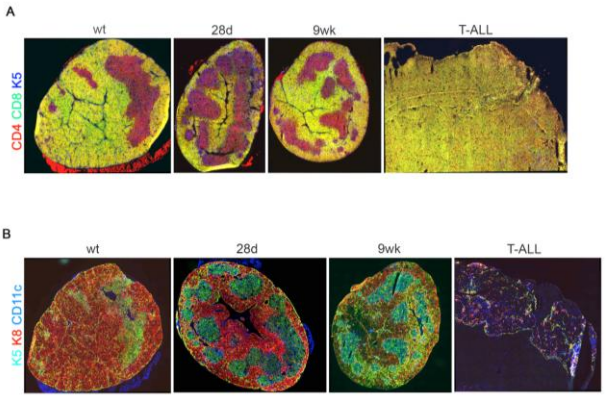

Figure 5- Progression from thymus autonomy to leukemogenesis has an impact in T cell development and epithelium. Wild type newborn thymi were grafted under the kidney capsule of adult recipients (wild type or *Rag2*$^{-/-}\gamma c^{-/-}$, as indicated) and analyzed 28 days, 9 weeks later and a timepoint in which T-ALL already developed by immunohistology for the indicated markers. (A) Staining for K5 (blue), CD4 (red) and CD8 (green). (B) Staining for K5 (green), K8 (red) and CD11c (blue). Images were acquired in Nikon High Content Screening microscope with the software Nikon Elements for the overview of the sections, using a 20x magnification.

Thymus architecture: Progression of thymus autonomy in a *Rag2*$^{-/-}$ background

T-ALL developed from wild type donor thymi as a consequence of prolonging thymus autonomy. Furthermore, T-ALL also developed from *Rag*$^{-/-}$ donor thymi, indicating that *Rag* is not necessary for leukemogenesis [5]. Since the transcriptome and the immunophenotype were identical between wild type and *Rag*-deficient T-ALL, we set to characterize the thymus of *Rag*$^{-/-}$ donors progressing towards T-ALL. Earlier timepoints, corresponding to those in which we study thymus autonomy in wild type thymi were also of interest to address, to characterize the parallel process in *Rag*$^{-/-}$ thymi. For this purpose, *Rag2*$^{-/-}$ thymi were transplanted under the kidney capsule of *Rag2*$^{-/-}\gamma c^{-/-}$ mice. Similarly, to the previous transplantation experiments, different timepoints were studied including 14, 28 days and 9 weeks after transplant (Fig. 6A).

In *Rag2*$^{-/-}$ thymus there are no medullary areas, and K5 is expressed in many epithelial cells, scattered through the thymus (Fig. 6B). Although some cells co-express K5 and K8, the majority of the epithelial cells only express either K5 or K8. The dendritic cells are also scattered throughout the organ. In all timepoints studied the lack of medullary areas and scatter of the DCs is constant and similar to the *Rag2*$^{-/-}$ control. Fourteen days after transplant, one of the grafts has a similar phenotype as the *Rag2*$^{-/-}$ thymus section, while in the other graft the majority of the cells are $K5^{+}K8^{+}$. Analyses of the grafts from 28 days and 9 weeks post-transplantation show the same heterogeneity as in day 14 after transplant, in which some grafts have TECs expressing K5 or K8 individually, while in others the tissue is marked by $K5^{+}K8^{+}$ cells (Fig. 6B). This can be better observed in the examples selected to show the complete thymus section. In general, the phenotype of the epithelium is maintained at every timepoint analyzed for the thymi in which thymocytes were still present (Fig. 6C).

Since *Rag2*$^{-/-}$ thymocytes cannot rearrange the TCRβ cannot progress beyond the double negative 3 stage. Therefore, no $CD4^{+}CD8^{+}$ double positive or single positive thymocytes could be detected in the thymus sections. As expected, the staining for K5 was scattered throughout the section, as no medullary areas organize in these thymi. In the thymus grafts (into *Rag2*$^{-/-}\gamma c^{-/-}$ recipients), this phenotype is maintained by 14 days post transplantation. Surprisingly, 28 days after transplant half of the grafts analyzed have double positive cells. No single positive thymocytes were detected. By 9 weeks after transplant, all grafts analyzed presented cells co-expressing CD4 and CD8 (Fig. 7A). One example per timepoint was selected to show a complete thymus section and permit evaluating how the thymus evolves in the course from autonomy towards leukemia (Fig. 7B). The *Rag2*$^{-/-}$ thymus grafts are identical to thymi that were not grafted by day 14. By day 28 post-transplantation $CD4^{+}CD8^{+}$ double positive cells emerge in half of the thymus grafts analyzed, and by 9 weeks after transplant all grafts analyzed had double-positive cells (Fig. 7A).

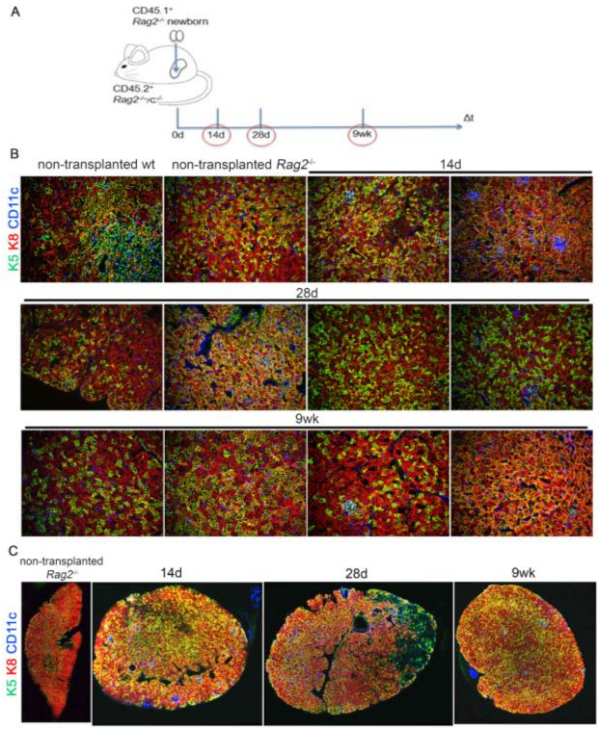

Figure 6- Thymic architecture in thymus autonomy from *Rag2*$^{-/-}$ donors. (A) *Rag2*$^{-/-}$ newborn thymi were transplanted under the kidney capsule of *Rag2*$^{-/-}\gamma c^{-/-}$ mice. The grafts were analyzed 14 days, 28 days and 9 weeks later. Wild type B6 and *Rag2*$^{-/-}$ thymus were used as staining control. (B,C) Staining for K5 (green), K8 (red) and CD11c (blue). Images were acquired in Leica DMRA2 microscope with the software MetaMorph using a 20x magnification. (C) Images were acquired in Nikon

High Content Screening microscope with the software Nikon Elements, using a 20x magnification.

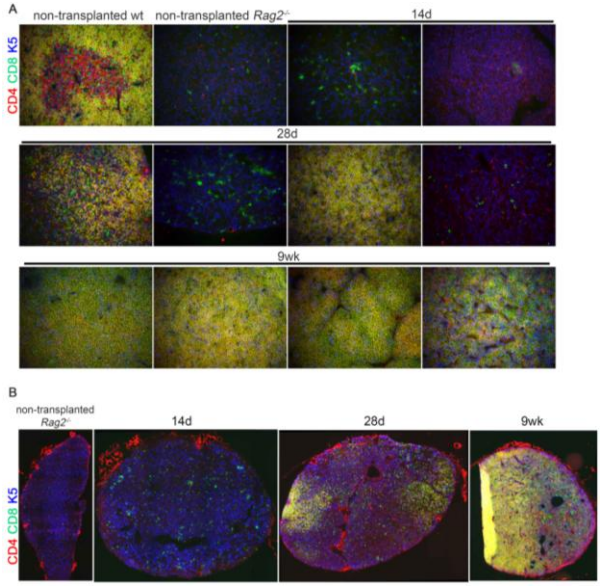

Figure 7- Double positive-like cells appear as thymus autonomy progresses in time. Sections of the same grafts shown in Figure 6 were stained for CD4 (red), CD8 (green) and K5 (blue). (A) Images were acquired in Leica DMRA2 microscope with the software MetaMorph using a 20x magnification. (B) The same samples in A were also acquired in a Nikon High Content Screening microscope with the software Nikon Elements, using a 20x magnification and one representative example of every timepoint is shown.

Thymus architecture: Thymus autonomy in multiple IL-7-unresponsive hosts

While deficiency in *Rag2* causes a developmental block at the double negative 3 stage and T cells fail to be generated, a deficiency in IL-7 signaling has a different impact in T cell differentiation. T cell development progresses through the several differentiation stages but leads to a marked decrease in the numbers of thymocytes. This leads to a dramatic decrease in the number of T cells produced. Former and current work by our lab shows that thymus autonomy can also arise in different recipients, with different genotypes, as long as they are unable to respond to IL-7. To study the structure of the thymus using different recipient mice, wild type newborn thymi were transplanted under the kidney capsule of *IL-7rα*$^{-/-}$, $\gamma c^{-/-}$ and *Rag*$^{-/-}$*2IL-7rα*$^{-/-}$ mice. Thymus structure was assessed 9 weeks after transplant (Fig. 8A).

Autonomy that arises from *IL-7rα*$^{-/-}$, $\gamma c^{-/-}$ and *Rag*$^{-/-}$*2IL-7rα*$^{-/-}$ can be characterized by a lymphocyte distribution similar to a wild type case, with double positive cells localized in the cortex and single positive cells in the medullas (K5^{+} mTECs). The only differences are the enrichment of CD8^{+} single positive cells in *IL-7r*$^{-/-}$ hosts (Fig. 8B).

Thymic epithelial cells and dendritic cell distribution is similar in *IL-7rα*$^{-/-}$, $\gamma c^{-/-}$ and *Rag*$^{-/-}$*2IL-7rα*$^{-/-}$ recipients. As previously reported with *Rag2*$^{-/-}\gamma c^{-/-}$ recipients, these grafts present a good separation between cortex and medullas, with DCs mainly localized in the medullas. Additionally, in the majority of the grafts, some cTECs co-express K5^{+}K8^{+}. (Fig. 8C).

Overall, the grafts analyzed from all 3 recipients resemble the phenotypes of thymus autonomy in *Rag2*$^{-/-}$ $\gamma c^{-/-}$ mice, indicating that the genotype of the host cells, as long as they cannot respond to IL-7, does not greatly impact on T cell development or thymic architecture during thymus autonomy.

Discussion

This work evaluated the architecture of the thymus in two extreme conditions: 1) in primary immunodeficiencies caused by defects of T lymphocyte development, and 2) during the progression that occurs from thymus autonomy to leukemia. The architecture of the thymus, i.e. the broad organization of the major cellular components of the thymus, was assessed by histology and focused on the composition and distribution of thymocytes, of TEC, and of dendritic cells. It was important to define the structure of the thymus in the immunodeficient mutants, as several were also used as recipients for the development of T-ALL.

As expected, all thymi with defects in the differentiation of T lymphocytes were dramatically reduced in cell numbers. While mutants for IL-7 or its receptor displayed a roughly normal distribution of thymocytes and TEC, Rag mutants had no cortex/medulla segregation [8][9]. This reflects the crosstalk that exists between developing T lymphocytes and TECs differentiation. *Rag2*$^{-/-}$ T cell precursors are unable to go beyond the double negative 3 stage during T lymphocyte development [10]. Accordingly, medullary areas are absent in the *Rag2*$^{-/-}$ thymus. This implicates that double positive and/or single positive thymocytes are required for the development of medullas. In the wild type thymus, TECs expressing both K5 and K8 are almost completely restricted to the cortico-medullary junction and identify a population of TEC progenitors [7]. As for *IL-7*$^{-/-}$, *IL-7rα*$^{-/-}$ and $\gamma c^{-/-}$ thymi, the normal distribution of thymocytes is kept, and a good separation between cortical and medullary areas was observed. Nonetheless, a significant population of TECs in the

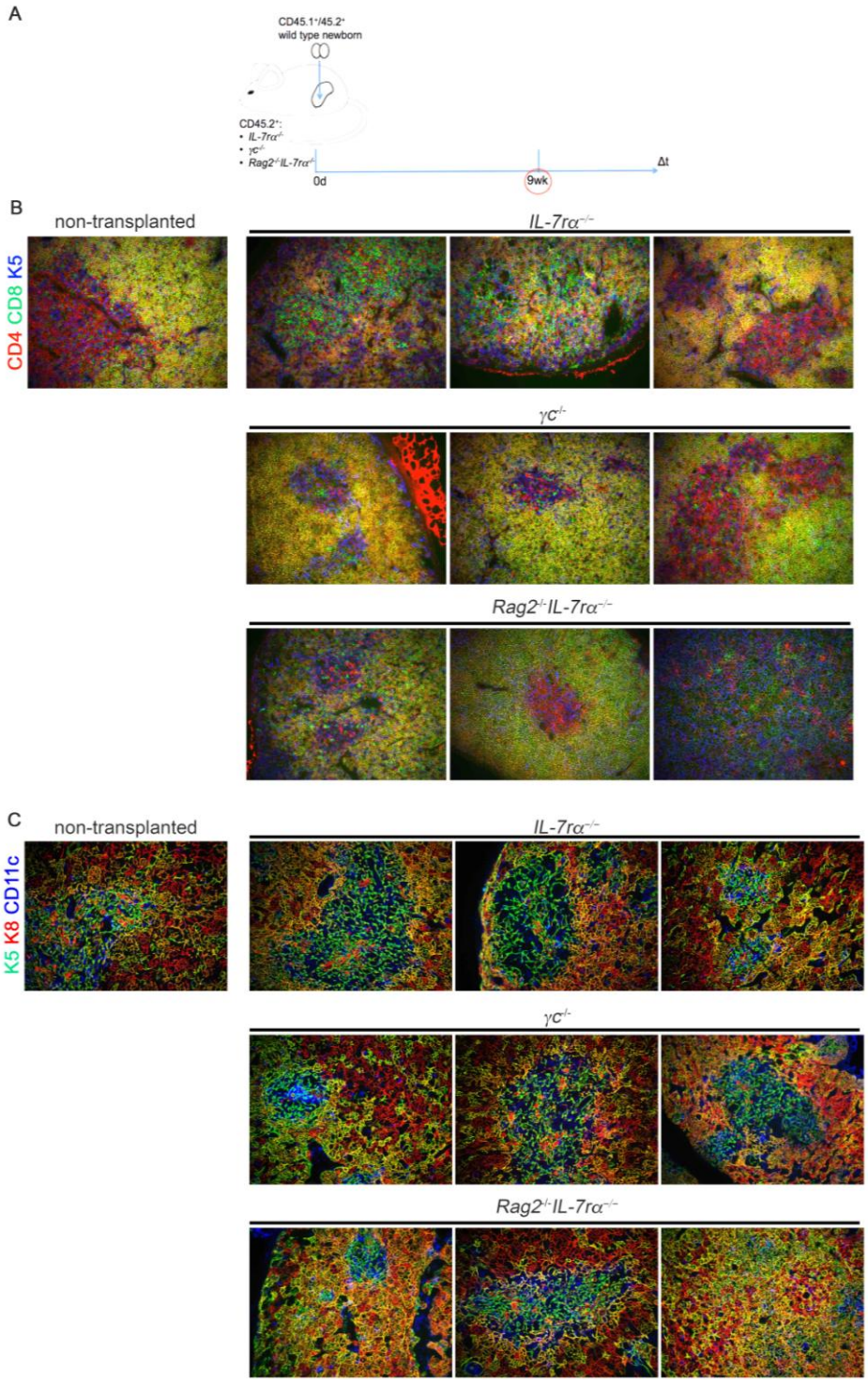

Figure 8- Thymus autonomy occurs from wild type donors grafted into *IL-7rα*$^{-/-}$, *γc*$^{-/-}$ and *Rag2*$^{-/-}$*IL-7rα*$^{-/-}$ recipients. (A) Representative scheme of the transplant model and timepoint studied using other recipients. Wild type B6 newborn thymi were transplanted into the following recipients: *IL-7rα*$^{-/-}$, *γc*$^{-/-}$ and *Rag2*$^{-/-}$*IL-7rα*$^{-/-}$ mice. (B, C) The grafts were analyzed 9 weeks later by immunohistology for the indicated markers. Wild type B6 non-transplanted thymus was used as staining control. (B) Staining for CD4 (red), CD8 (green) and K5 (blue). Images were acquired in Leica DMRA2 microscope with the software MetaMorph using a 20x magnification. (C) Sections of the same grafts shown in B were stained for K5 (green), K8 (red) and CD11c (blue). Images were acquired in Leica DMRA2 microscope with the software MetaMorph using a 20x magnification. This experiment was performed with 13 mice.

cortical areas co-expressed K5 and K8, which is reminiscent of an immature TEC phenotype, possibly resulting from the reduced thymocyte number present in the cortex.

The architecture of the thymi in which thymus autonomy was active was identical to the normal thymi, in which progenitor colonization followed by turnover takes place at early stages post transplantation. However, with prolongation of autonomy in time TEC architecture starts to change. Specifically, cTECs begin to express both K5 and K8, consistent with the reduction of thymocyte number in these thymi. Interestingly, in the thymus grafts where donor T cell development ceased, the vast majority of cTECs co-expressed K5 and K8 and no lymphocytes were present in the cortex. Circulating T lymphocytes located in the medullary areas in such cases.

T-ALL eventually emerged, and leukemic cells co-expressed CD4 and CD8 to variable degrees. These accumulated in the thymus grafts causing tissue destruction that was obvious from the complete disruption of the network usually displayed by TEC.

Thymus autonomy occurs in *IL-7rα*$^{-/-}$, *γc*$^{-/-}$, *Rag2*$^{-/-}$*IL-7r*$^{-/-}$ and *Rag2*$^{-/-}$*γc*$^{-/-}$ recipients. Since the hematopoietic progenitors in these mice differ in phenotype and in their capacity to respond to cytokines, it was interesting to compare whether that had any impact on thymus structure. No differences were detected between these recipients by 9 week-post-transplantation. Therefore, IL-7 is the determinant factor for thymus autonomy while other γc cytokines have no impact in this process.

T-ALL also emerges from *Rag2*$^{-/-}$ donors [5]. Under the assumption that T-ALL is preceded by a period of thymus autonomy we confirmed that the phenotype of the *Rag2*$^{-/-}$ thymus is kept for some time, both in thymocytes and TEC. Thymi were characteristically devoid of medullary areas, the dendritic cells were scattered and most TEC expressed either K5 or K8 without an obvious organization. As soon as by 28 days post transplantation, thymocytes in half of the autonomous thymi co-expressed CD4 and CD8, which normally would never occur in *Rag2*$^{-/-}$ thymocytes. Nine weeks later, all thymi displayed the same phenotype. It is highly suggestive that this corresponds to a pre-leukemic state. This is similar to experiments of *Rag*-deficient mice exposed to sublethal doses of γ-radiation and developed double positive-like cells afterwards. The authors propose the existence of alternative pathways to β-selection that might enforce the differentiation from double negative to double positive in conditions of stress [11]. Although the experimental setup is not the same, it is conceivable that cells in thymus autonomy are under stress caused by self-renewal and excess proliferation. These could be responsible for the forced differentiation of the thymocytes into a double positive state.

In sum, thymus architecture is kept roughly normal during autonomy for the time in which thymopoiesis is normal but is reshaped over time as result of reduction of cellularity. The normal structure disappears in the leukemias, and the thymus is fully destroyed.

Materials and Methods

Mice

All mice used for the work described here were bred and kept at the mouse facility of Instituto Gulbenkian de Ciência (IGC). Strains that were kept and produced at the IGC were the C57BL/6J (B6, CD45.2^{+}), *IL-7*$^{-/-}$ and *Rag2*$^{-/-}$. The following strains were purchased from The Jackson Laboratory prior to establishing the respective colonies at IGC: B6.SJL-*Ptprc*a Pep3b/BoyJ (CD45.1+), *IL-7rα*$^{-/-}$ and *γc*$^{-/-}$. *Rag2*$^{-/-}$ and *IL-7rα*$^{-/-}$ mice were crossed to generate the *Rag2*$^{-/-}$*IL-7rα*$^{-/-}$ line. *Rag2*$^{-/-}$*γc*$^{-/-}$ mice were imported from Ulm, Germany, and rederived. All mice were bred and maintained in individually ventilated cages (IVCs) and in the specific pathogen free (SPF) area of the mouse facility. All animal experiments described here were approved by the Ethics Committee of the IGC – Fundação Calouste Gulbenkian and the Direção Geral de Alimentação e Veterinária (DGAV).

Thymus transplants

The thymi from newborn F1 donor mice (B6xB6.SJL) were isolated and the lobes were separated and kept in cold PBS. The recipient mice were anesthetized with Ketamin (100mg/kg) and Xylazine (16mg/kg). The surgery was performed in fully anesthetized animals (after pressing the paw to ensure that the mouse could not feel pain). Each recipient mouse received one thymus, transplanted under the kidney capsule, being each thymus lobe placed in each extremity of the kidney. All transplants were executed by Rafael Paiva, a PhD student from the Lymphocyte Development and Leukemogenesis laboratory that is certified to do so.

Histology

Thymus grafts were isolated from the recipient mice at the specified time points and cryopreserved in OCT. The frozen blocks were stored at -80ºC until further use. The grafts were sectioned into 8 µm-thick slices by the Histopatology Unit of the IGC, using a Leica Cryostat CM 3050 S. The sections were collected onto glass slides, air dried and dehydrated in acetone for 10 minutes. The dehydrated and dried slides were stored at -80ºC.

Immunohistology

To proceed with the staining of the thymus sections, the slides were taken into new boxes in the cold to maintain the humidity-free environment. These were then allowed to reach room temperature. The sections were isolated using a PAP pen and rehydrated in PBS for at least 5 minutes. All blocking and staining steps were done in PBS/10%FBS in a humid environment. Blocking of the sections was performed together with the staining for DAPI (Sigma) with 114µg/ml mouse IgG (Jackson Laboratories) during 30 minutes. The staining with primary antibodies was carried out overnight at 4ºC. After washing 3 times for 5 minutes, sections were incubated for 30 minutes, at room temperature, with the secondary antibodies. All antibodies were purchased from Biolegend. The primary antibodies used were rabbit polyclonal anti-mouse Keratin 5 (Poly19055), mouse anti-Keratin 8-Alexa647 (1E8) and hamster anti-CD11c-PE (N418), or rat anti-CD4-bio (GK1.5) and rat anti-CD8-APC (53-6.7). The secondary antibodies used were α-Rabbit Alexa488 and Streptavidin-Cy3. After the incubation, the slides were washed again 3 times for 5 minutes. Stained slides were mounted with Fluoromount

G (both from Southern Biotech and Invitrogen) and kept cold prior to image acquisition.

Microscopy

The images were acquired with a 20x magnification in both Leica DMRA2 microscope using the software MetaMorph and Nikon HCS microscope using Nikon Element, at the Advanced Imaging facility of the IGC. The image treatment was performed using FIJI/ImageJ and Adobe Photoshop CC 2018.

Flow cytometry

Organs were harvested and single-cell suspensions were prepared in cold PBS/10% FBS. Cells were counted with trypan blue exclusion using a Neubauer chamber. The cells were blocked with 11 g/ml mouse IgG (Jackson Laboratories) for 15 minutes and then stained for 30 minutes with the following antibodies: hamster anti-CD3ε APC-Cy7 (45-2C11), rat anti-CD4 PE (GK1.5), rat anti-CD4 PE-Cy7 (GK1.5), rat anti-CD8 FITC (from the Antibody Service of IGC, YTS169.4), rat anti-CD25 BV605 (PC61) and mouse anti-CD44 PerCP-Cy5.5 (A20). SYTOX blue was used to exclude dead cells (Molecular Probes). All antibodies were purchased from Biolegend, except for CD8-FITC, which was produced and labelled at the Antibody Service of the IGC. The samples were acquired on a BD Fortessa X-20 cell analyzer using a BD FACSDiva 8 software. Final analysis was performed using FlowJo.

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