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Complex and Multilayered role of IL-21 signaling during thymic development

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Abstract

Unlike IL-7, which is known to be critical for T cell thymic development, the role of IL-21 in this process is still controversial. IL-21 has been shown to accelerate thymic recovery in mice treated with glucocorticoids and revives the peripheral T cell pool in aged animals. However, mice with a defect in IL-21 signaling exhibit normal thymic cellularity, challenging the importance of this cytokine in the thymic developmental process. Using mixed bone marrow chimeric mice, our studies describe a multilayered role for IL-21 in thymopoiesis. In this system, IL-21 receptor-deficient cells are unable to compete with WT populations at different stages of the thymic development. Using a mixed bone marrow chimeric animal model, IL-21 seems to be involved as early as the DN1 stage and the cells from the knock out compartment have problems transitioning to subsequent DN stages. Also, similar to IL-7, IL-21 seems to be involved in the positive selection of DP lymphocytes and appears to play a role in the migration of single positive T cells to the periphery. Although not as critical as IL-7, based on our studies, IL-21 plays an important complementary role in thymic T cell development which, to date, has been under-recognized.

Introduction:

The thymus provides a unique environment for the development and maturation of T cells. T cell lymphopoiesis is responsible for maintaining a pool of naive peripheral T cells with a broad spectrum of TCR specificities. On the basis of CD4 and CD8 T cell expression, thymopoiesis can be broadly divided into three major stages, namely, double negative (DN), double positive (DP), and single positive (SP) cells. The key events during this process include the entry of lymphoid progenitor cells originating from the bone marrow, the formation of a functional T cell receptor (TCR) through TCRβ chain and α chain rearrangement and positive and negative selection to ensure major histocompatibility complex (MHC) restriction as well as clearance of autoreactive cells (1).

The role of several common γ chain cytokines in the thymopoiesis process is well appreciated. Amongst them, IL-7 is essential for lymphocyte development and survival.

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Mice deficient in IL-7 and IL-7Rα exhibit significant reductions in T and B cells (2, 3). Specifically, IL-7 is critical for early lymphocyte development by supporting proliferation, survival, and differentiation of DN subset (4). Additionally, differentiation of positively selected CD8 T cells in the thymus is contingent on IL-7 signaling (5, 6). Similarly, two other common γ chain cytokines, IL-15 and IL-2, have been reported to be involved in regulatory T cell thymic development (7). In a study conducted a few years ago, it was also demonstrated that IL-18, in synergy with IL-7, can promote bone marrow lymphopoiesis and T cell development (8).

IL-21 is one of the most recently characterized members of the common γ chain cytokine family (9). It is produced primarily by activated CD4 T cells in the periphery and epithelial cells in the thymus (10, 11). The cytokine is involved in a number of functions which includes, promoting CD4 differentiation, co-stimulation of activated NK cells, and IgG production by B cells (11–13). IL-21R, which is expressed by all lymphocytes, forms a heterodimer with the shared common γ chain subunit (9). IL-21, unlike IL-7, is not considered to be essential for thymopoiesis as IL-21R KO animals exhibit normal thymic cellularity (14). However, it has been reported that IL-21 treatment of mice with glucocorticoids-induced thymic atrophy, significantly accelerates the recovery of thymic functions (15). Moreover, in a very recent study, it was demonstrated that the peripheral T cell pool of aged animals was rejuvenated by administration of IL-21 (16). This could be explained by the ability of IL-21 to induce expansion of bone marrow-derived hematopoietic progenitor cells (17, 18). Furthermore, a recent *in vitro* study conducted by Rafei *et al.* demonstrated that IL-21 has the unique ability to up-regulate BCL-6, expand DP thymocytes undergoing positive selection, and increase the production of mature T cells (10). Additionally, this study showed that, in contrast to IL-7 (5), CD8 T cell differentiation was IL-21-independent. These observations reveal the complex role of IL-21 in enhancing the thymic T cell output in aged or disease-related thymic atrophy.

In this study, we observed that, although IL-21 expression in the thymus was significantly lower than IL-7 and IL-15, every single thymic subset expressed the IL-21R. Considering that normal thymic cellularity in IL-21R KO mice may be attributed to a redundant mechanism(s), we decided to investigate the role of IL-21 in thymic T cell development using WT: IL-21R KO mixed bone marrow chimeric mice. In this model, lack of IL-21 signaling led to various defects, starting as early as the DN1 stage and involved all the subsequent DN stages. Effectively, *in vitro* coculture of DN1 cells with IL-7 and IL-21 showed greater differentiation than those treated with IL-7 alone. Additionally, the frequency of the more mature DP population was reduced in the knockout compartment of the mixed bone marrow chimeric mice. Emigration of single positive CD4 and CD8 T cells may also be affected by lack of IL-21 signaling as these cells expressed lower expression of S1P1R than WT counterparts and exhibited reduced migration to S1P in a transwell migration assay. These findings suggest a complex supplementary role for IL-21 in thymic development that stretches beyond survival and expansion of the different thymic subsets.

Material and Methods:

Mice

C57BL/6, Thy1.1, and CD45.1 congenic mice were obtained from The Jackson Laboratory. IL-21R KO mice (B6N.129-Il21r<tm1Kopf>/J), originally purchased from The Jackson Laboratory, were bred in house under specific pathogen free conditions at the Animal Research Facility at The George Washington University (Washington, DC). Mixed bone marrow chimeric animals (WT: IL-21R KO at a 1:1 ratio) were generated as previously described (19). Briefly, lethally irradiated Thy1.1 recipients (8Gy/20g of body weight) were injected intravenously with $5x10^6$ magnetically purified hematopoietic progenitor cells from WT (CD45.1) and IL-21R KO (CD45.2) animals (Stemcell). Transferred animals received water supplemented with sulfamethoxazole and trimethoprim (Hi-Tech Pharmacal Co) for 4-5 weeks after injection and experiments were conducted 4-8 weeks post reconstitution. All animal experiments were approved by The George Washington University School of Medicine and Health Sciences Institutional Animal Care and Use Committee.

Quantification of common γ chain cytokines by real time PCR

IL-2, IL-7, IL-15 and IL-21 were detected by real time PCR using previously described primers and conditions (19). RNA was isolated from spleen and thymus with Trizol reagent (Thermofisher), and cDNA was generated using MMLV reverse transcriptase. Amplification was performed with a myIQ thermocycler (BIO-RAD) for 40 cycles comprised of 95°C for 45 sec, 61.4°C for 50 sec and 72°C for 45 sec. Efficiency for each set of primers was verified using a 2 fold dilution series (20). Gene expression of common γ chain cytokines was normalized using β actin as endogenous control.

Flow cytometry

Antibodies used for flow cytometry analysis were purchased from Biolegend [CD45.1 (A20), TCRγδ (GL3), B220 (RA3-6B2), Notch1 (HMN1-12), CCR7 (4B12), CD62L (Mel. 14)] and ThermoFisher Scientific [CD45.2 (104), Thy1.1/CD90.1 (HIS51), CD4 (GK1.5), CD8 (53.6.7), CD3 (145-2C11), Gr1 (RB6-8C5)), CD44 (IM7), CD25 (PC61.5), C-Kit (2B8eBio), CD27 (LG.7F9), IL-21R (eBio4A9), CD5 (53-7.3), CD24 (M1/69), CD69 (H1.3F3)]. S1P1 (713412) was purchased from R&D Systems.

An antibody cocktail (containing anti-CD4, -CD8, -CD19, -B220, -NK1.1, -CD11b, -CD11c, -TCRβ, -TCRγδ, and -Ter119) was used for exclusion of lineage negative thymocytes in order to facilitate the detailed analysis of the various DN subsets.

Cell suspensions were prepared and labeled as previously described (19). Staining for CCR7 was performed at 37°C according to manufacturer's instructions (Biolegend). Data were acquired using a FACS Calibur flow cytometer with a Cytek upgrade (Becton Dickinson, Cytek Development Inc). Analysis was carried out with FlowJo software (Flowjo, LLC).

Annexin V detection was performed as followed. Briefly, cells were isolated from thymus and spleen of mixed BMC as described above and rested 4 hours at 37°C prior to annexin V staining in accordance to manufacturer's recommended protocol (Biolegend).

S1P migration assay

Migration of thymocytes in response to S1P was assayed as previously described (21). Thymocytes from WT or IL-21R KO mice were serum starved for 2 hours and then treated with migration media (Iscove modified Dulbecco medium supplemented with 1100μg/ml delipidated bovine serum albumin (Sigma-Aldrich), 2mM L-glutamine, and 25 μg/ml penicillin/streptomycin) containing FTY720 or not (100nM, Sigma-Aldrich) for 1 hour at 37°C and 5% CO2. Briefly, $2.5x10^6$ thymocytes were added in the upper chamber of a transwell plate (Corning Costar, 5μm pore size) and different concentration of S1P (10, 100, 1000 nM, Sigma-Aldrich) or migration media control were added to the lower chamber. Cells were allowed to migrate for 3 hours at 37°C and 5% CO2. Cells from the bottom chamber were then harvested and stained before acquisition on BD Celesta. CountBright™ Absolute counting beads (ThermoFisher Scientific) were used to determine the exact number of cells migrated for each condition. Migration index was calculated as follow: number of cells recovered from the bottom chamber / number of cells seeded in the top chamber x 100.

OP9 coculture

Differentiation of DN subsets in vitro was performed as previously described (22). Briefly, lin^- population was enriched by negative magnetic selection before DN1 cells ($CD44^{hi}CD25^{lo}$) were sorted by flow cytometry (purity≥90%). DN1 cells ($5x10^3$ cells/well) were incubated with the OP9-DL1 thymic stromal cell line with either murine recombinant IL-7 (5ng/ml) (Peprotech), murine recombinant IL-21 (100, 50 or 10 ng/ml) (Peprotech), IL-7 + IL-21 (5ng/ml and 50ng/ml respectively) or control with media alone. Cells were passed onto a fresh OP9-DL1 monolayer complemented with the appropriate cytokine treatment every 3 days. Cells were maintained in culture for 10-12 days before harvesting and staining for flow cytometry analysis.

BrdU incorporation assay

In vivo proliferation of lymphocytes from spleen of thymus was assessed by bromodeoxyuridine (BrdU) incorporation assay. One group of mixed BMC mice was orally infected with $2x10^7$ *Encephalitozoon cuniculi* spores (genotype III maintained as previously described (23)). All mice were injected intraperitoneally every other day with 1mg BrdU/ mouse as previously described (19), starting 7 days prior to sacrifice. Detection of intranuclear BrdU incorporation was performed according to manufacturer's instruction (BD Pharmingen).

Statistical analysis

Results are presented as mean ± s.d. Comparison between groups was performed by Student's *t*-test throughout the study.

Results:

Common γ chain cytokines levels in WT and IL-21R KO mice:

The role of IL-7 in thymopoiesis has been extensively studied and both IL-7 KO and IL-7R KO animals display severe T cells defect (2, 3). On the other hand, the involvement of IL-21, another member of the common γ chain cytokine family which mediates its functions through a heterodimeric receptor composed of IL-21R and the common γ chain receptor (9), seems to be more controversial. Early northern blot analysis determined that IL-21R expression is restricted to lymphoid tissues, namely spleen and thymus (9, 24). However, IL-21R KO mice display normal cellularity in both of these tissues (14). Therefore, mRNA levels of different common γ chain cytokines in the thymus and spleen from C57Bl/6 mice were analyzed. As expected, IL-7 and IL-15 are the most predominant members in both thymus and spleen, and as compared to IL-7, IL-2 and IL-21 expressions were significantly lower in both tissues (figure 1A). Although levels of IL-7 and IL-15 mRNA in the tissues (thymus and spleen) of IL-21RKO mice were decreased compared to the WT animals, the expression for both cytokines was still substantial (figure 1A). Also, IL-21 mRNA expression was significantly reduced in the spleen of the KO mice. Next, expression of IL-21R by different thymic populations was assessed by flow cytometry. As shown in figure 1B, similar to previous findings (15, 25), all subsets in the thymus (DN, DP, SP CD4 and SP CD8) expressed IL-21R even though the expression was lower in SP CD4 thymocytes as compared to SP CD8 cells. Low expression of IL-21 in thymus and broad expression of IL-21R by thymic subsets suggest an ambivalent role for IL-21 in thymopoiesis.

Lack of IL-21 signaling leads to suboptimal thymopoiesis in a mixed bone marrow chimeric model:

Several recent reports showed that exogenous administration of IL-21 leads to an increase in thymus size and cellularity (16, 17), suggesting that this cytokine is involved in the development of thymocytes. However, these results were undermined by the fact that IL-21R KO mice exhibit normal T cell and B cell development (14). To better decipher the intrinsic role of IL-21 in the development of different thymic populations, a mixed bone marrow chimeric (BMC) strategy was used (WT: IL-21R KO at a 1:1 ratio). At 2 months post-reconstitution, the frequency of IL-21R KO cells in thymus was significantly lower than the WT counterpart (figure 2A). The difference was also detected in the spleen of these animals, although the frequency of IL-21R KO cells decreased earlier in the spleen than in the thymus. The absence of IL-21 signaling also affected the distribution of the different populations in both thymus and spleen. As shown in figure 2B, compared to WT cells, frequencies and total numbers of IL-21R KO DN, CD4, and CD8 subsets were reduced in the BMC mice. Due to the reduction in total number of IL-21R KO cells in BMC mice, we also observed a significant decrease in the total number of DP in these animals. As compared to WT cells, splenic IL-21R KO CD4 and CD8 populations were also significantly decreased in these animals. The frequency and total number of γδ T cells, which arise from immature DN thymocytes that have rearranged the TCRγ and TCRδ loci (26), were also significantly lower in the spleen and thymus from the IL-21R compartment compared to WT counterparts (figure 2C). However, the frequency of B cells, eosinophils,

macrophages and monocytes (which includes DC in our gating strategy) in the spleen were not affected by lack of IL-21 signaling (figure 2D).

Development of DN in IL-21R KO compartment of mixed bone marrow chimeric animals:

Since the DN population was reduced in the IL-21R KO compartment from BMC mice (figure 2B), next, we investigated the earlier stages of the thymic development in these animals. Based on CD44 and CD25 expression by lineage negative population (lin^- as defined in material and methods section), DN can be subdivided into DN1, DN2, DN3 and DN4 stages (27). However, but the distribution of the WT and IL-21R KO cells between these subsets is somewhat different in a chimeric environment and is similar to previously published reports (28, 29). Interestingly, the percentage as well as the total number of DN1 cells ($CD44^{hi}CD25^{lo}$) were significantly higher in IL-21R KO lin^- population as compared to WT compartment of the BMC, while subsequent development stages (DN2-4) were lower amongst this population (figure 3A). However, the identification of DN1 stage with CD44 and CD25 may not be sufficient since the $CD44^{hi}CD25^{lo}$ subset is comprised of pluripotent early thymic precursor (ETP) along with other cell types (30). ETP and DN2 subsets are defined by high expression of the stem cell factor receptor, c-Kit (31). A substantial increase in thymic precursors ($CD44^{hi}$c-Kit^{hi}) within the lin^- population was detected in the absence of IL-21 signaling (figure 3B). As shown in figure 3C, the frequency of ETP was 2 to 3-fold higher in the IL-21R KO $CD44^{hi}$c-Kit^{hi} population as compared to WT counterpart, while the DN2a and DN2b subsets were significantly reduced in the KO compartment (32).

Subsequently, cell size and CD27 were used to evaluate the subdivision of DN3 into DN3a (pre-β selection cells) and DN3b (post-β selection cells) (33). Our data revealed that both frequency and total number of DN3b ($FSC^{hi}CD27^{hi}$) were significantly lower in IL-21R KO lin^- population of BMC as compared to WT counterparts (figure 3D). Subsequently, we determined if the defect observed in IL-21R KO compartment during early thymic stages could be attributed to dysfunctional Notch signaling pathway since this transcription factor has been reported to play a critical role in the early T cell lineage commitment (34). Interestingly, expression of Notch 1, which has been shown to be both necessary and sufficient for T cell development (34, 35), was higher in the DN subset from the IL-21R KO compartment, as compared to WT counterpart (figure 3E). However, Notch 2 expression was similar between DN from WT and IL-21R KO compartment (data not shown). The role of IL-21 in DN1 differentiation was further assessed *in vitro* using the stromal cell line OP9 expressing the DL1 receptor for Notch (OP9-DL1) (22). For this purpose, sorted DN1 cells were cocultured with OP9-DL1 in presence of IL-21, IL-7 or IL-7 + IL-21. As shown in figure 3F, after 10-12 days of coculture, sorted DN1 cells differentiated into DN2 and DN3 after treatment with IL-7 and IL-7 + IL-21. However, IL-21 seemed to have an additive effect as percentage of DN1 remaining in culture were lower when the cells were treated with both IL-7 and IL-21 as compared to those treated with IL-7 alone. Interestingly, IL-21 alone did not promote differentiation to the DN2 or DN3 subsets within the time frame of this experiment even though CD25 expression was significantly increased with higher concentrations of IL-21 (figure 3F). From these observations, it can be postulated that, in the absence of IL-21 signaling, DN1 development seems to be impaired and processing to the subsequent stages (DN2 and beyond) seems to be significantly compromised.

Role of IL-21 signaling in thymic selection and maturation of single positive cells:

It has been reported by Haks *et al.* that intrinsic properties of the TCR-CD3 complex regulate the selection process at the DP checkpoint (36). Effectively, signal strength through the TCR complex on DP thymocytes regulates the positive and negative selection signals, which lead to the upregulation of surface proteins CD69 and CD5 (37). Therefore, expression of these receptors can be used to monitor activation and maturation at the DP stage. Our data show that IL-21 signaling is important for the maturation of the DP population as cells from the IL-21R KO compartment exhibit a less mature phenotype with a frequency of post-selection DP thymocytes ($CD3^{hi}CD5^{hi}$) significantly lower than WT cells (figure 4A). Also, CD5 expression by DP from IL-21R KO compartment was significantly lower than WT counterparts (figure 4A). These observations were further emphasized by analysis of CD3 and CD69, which demonstrated that both the early post-positive selection ($CD3^{hi}CD69^{hi}$) and the more mature ($CD3^{hi}CD69^{lo}$) populations were reduced in the IL-21R KO compartment (figure 4B). The role of the chemokine receptor CCR7 in thymic maturation is well appreciated and is important for the movement of thymocytes from the cortex to the medulla (38). Interestingly, no difference in the expression of CCR7 by WT and IL-21R KO DP populations from BMC animals was noted (figure 4C).

Once in the medulla, newly generated SP CD4 and CD8 T cells are functionally immature and express high levels of CD24, a heat stable antigen (39), During the final maturation steps, single positive CD4 and CD8 T cells downregulate CD24 and CD69 before gaining access to the periphery (40). Flow cytometry analysis indicates that the maturation of SP CD4 and CD8 T cells isolated from the thymus of mixed BMC mice is not affected by the lack of IL-21 signaling as no significant differences were noted between the WT and KO compartment (figure 5A). SP CD4 and CD8 T cells exhibit high expression of CD62L and S1P1R, which is facilitated by transcription factor KLF2 expression (41). Similar to the observations made with CD69, levels of CD62L and CD24 expression did not appear to be significantly different between the cells in IL-21R KO and WT compartments from the BMC mice. As shown in fig 5B, the frequency of single positive mature CD4 and CD8 T cells ($CD24^{lo}CD62L^{hi}$) were similar between the two compartments (figure 5B). Also, frequency of $CD3^{+}$ cells amongst SP CD4 and CD8 T cells are comparable between WT and IL-21R KO compartments (data not shown). After mature SP thymocytes (CD4 and CD8) downregulate CD69 expression, the S1P1 receptor is retained on the surface of the cell, initiating thymic egress when S1P is detected in the vicinity of blood vessels (41). Importantly, both CD4 and CD8 T cells from IL-21R KO population are unable to express optimal S1P1R levels which likely compromises their ability to emigrate from the thymus to the peripheral circulation (figure 5C). To determine the efficiency of SP CD4 and CD8 T cells from WT and IL-21R KO mice to respond to S1P stimulus, a transwell migration assay was performed (21). As shown in figure 5D, both CD4 and CD8 T cells from IL-21R KO exhibit reduced migration to the lower chamber of the transwell at all the concentrations of S1P tested while cells from the WT animals respond to the stimulus. The impaired ability to migrate in response to S1P could contribute to the reduced frequency of IL-21R KO CD4 and CD8 T in the spleen of BMC animals. These studies demonstrate that, while IL-21 signaling does not seem to play a role in the maturation of SP CD4 or CD8 populations, it most likely is important for their emigration to the periphery.

IL-21 signaling is important for survival and expansion of different cell subsets both in the thymus and the spleen:

The role of IL-21 in promoting survival and expansion of T cells in antigen-dependent, as well as independent manner, has previously described (42, 43). Both CD4 and CD8 T cells arise from a very small number of early thymic progenitors, which expand greatly during the process of thymic development. As these cells also undergo apoptosis during the negative selection process, next, we determined the role of IL-21 in the expansion and survival of the different thymic subsets. At first, the survival of thymic subsets was measured by evaluating the expression of pro-apoptotic protein annexin V. As shown in figure 6A, the cell death pattern was different between WT and IL-21R KO populations in mixed BMC, as DN population of IL-21R KO compartment was more apoptotic than WT cells. Annexin-V expression in the DP population was not different between the two compartments. Interestingly, we observed an increased expression of annexin-V in the IL-21 KO CD8 T cells in the thymus (figure 6A) and the spleen (figure 6B) from the mixed BMC animals. Importantly, there was no difference in the expression of annexin-V in CD4 T cells between IL-21R KO and WT compartment in both thymus and spleen (figure 6A–B).

To determine the role of IL-21 in the proliferative ability of the different subsets from the thymus and spleen of BMC animals, we performed a BrdU incorporation assay. As shown in figure 6C, impaired IL-21 signaling triggered a slightly lower frequency of proliferative CD8 T cells in the thymus. Conversely, the frequency of thymic DN, DP and CD4 T cells from the IL-21R KO compartment with newly incorporated BrdU was similar to those in the WT compartment. Interestingly, as compared to the WT compartment, the proliferative ability of both CD4 and CD8 splenic T cells was impaired in the absence of functional IL-21 signaling (figure 6D).

To assess the role of IL-21 in the expansion of T cells in an infectious environment, mixed BMC animals were orally infected with *Encephalitozoon cuniculi*, a pathogen that induces a potent cytotoxic T cell response dependent on IL-21 (19). While no difference in BrdU incorporation between the WT and IL-21R KO compartment of the infected BMC animals was observed for any of the thymic subsets (DN, DP, CD4 and CD8) (data not shown), proliferative ability of both splenic CD4 and CD8 T cells lacking functional IL-21 signaling was significantly impaired in these animals (figure 6E). These studies demonstrate that IL-21 signaling is important for the survival of some thymic subsets (DN and CD8 SP) and the CD8 T cell population in the spleen. Also, this cytokine plays an important role in the expansion of the CD8 T cell population in both the thymus and the periphery.

Discussion:

The observations presented in this manuscript reveal a multi-layered role of IL-21 in the thymic development of T lymphocytes and demonstrate the involvement of IL-21 not only in the differentiation of the DN subsets but also in the maturation and emigration process of subsequent thymic populations. The fact that all thymic subsets (DN, DP, CD4, and CD8) express IL-21R, suggests a potential role for this cytokine in thymopoiesis. Using BMC mice, we observe that the IL-21R KO population is under-represented in both the thymus and spleen of chimeric animals. Moreover, this defect is detected as early as the DN1 stage

and is followed by a subsequent decrease in the generation of DN2-DN4 populations in the KO compartment. Although the expression of CCR7 by WT and IL-21R KO population is not different, IL-21 seems to be involved in the positive selection process. Also, based on our observations, IL-21 signaling does not seem to be important in the maturation of SP lymphocytes (CD4 and CD8), however, the lower S1P1 receptor expression, and diminished ability to migrate to S1P *in vitro* displayed by IL-21R KO cells therefore can compromise their migration to the periphery.

The thymic T cell development is known to be critically dependent on IL-7 (44). This cytokine, which is produced by thymic epithelial cells, plays a major role from the early stage of DN to differentiation of CD8 and TCR γδ intraepithelial lymphocytes (44). In absence of IL-7 signaling, the differentiation of immature thymocytes is compromised and IL-7R deficient cells are arrested at the DN3 stage (2, 3). However, the fact that mice with genetic deficiency in common γ chain cytokines, not including IL-7, display normal cellularity in both spleen and thymus has complicated the evaluation of their role in thymopoiesis (44). Therefore, based on this seemingly normal T cell development in IL-21R KO mice and the contradicting fact that IL-21 can improve thymic recovery during aging or after glucocorticoid treatment, a mixed BMC approach (WT and IL-21R KO) was used to determine the intrinsic role of this cytokine in the thymic development. We observed that the frequency of DN subset and SP T cells (CD4 and CD8) are reduced in the absence of IL-21 signaling. In addition, an increased frequency of DN1 population is detected in the IL-21R KO compartment, however, these cells do not transition efficiently to subsequent stages (DN2-DN4). As shown by OP9-DL1 and DN1 coculture, IL-21 contributes to the differentiation of DN1 cells into subsequent DN subsets although IL-7 plays a more predominant role in this process. Interestingly, our data reveal that IL-21 is involved as early as the DN1 stage, which is earlier than IL-7, as this begins only at the DN3 stage and coincides with TCRβ selection of DN thymocytes (4). Although the role of IL-21 in the proliferation and survival of T cells in the periphery has been documented (42, 43), the reduced frequency of DN population in IL-21R KO compartment can also be attributed to an increase in apoptosis rather than a defect in their proliferative ability. Also, similar to IL-7, IL-21 does not seem to be involved in the commitment of lymphoid progenitors to a T cell lineage as Notch expression was not decreased by lack of IL-21 signaling.

Although our studies highlight an important role for IL-21 in the development of DN stages, lack of IL-21 signaling also seems to affect the transition from post-positive selection ($CD3^{hi}CD69^{hi}$) into a mature population ($CD3^{hi}CD69^{lo}$) as the frequency of mature thymocytes is significantly lower in the IL-21R KO compartment. These data are consistent with *in vitro* studies performed by Rafei *et al* (10) showing a strong induction of IL-21R by DP thymocytes undergoing positive selection (10). However, conflicting with these studies (10), our report shows that IL-21R KO DP in mixed BMC exhibit a normal proliferative ability, as BrdU incorporation levels are similar to the cells from the WT compartment. The differences can be explained by the fact that we are performing *ex vivo* studies using BMC mice that addresses the role of IL-21 signaling in a competitive environment; while the data from the studies of Rafei *et al* were generated by treating WT thymocytes with IL-21 *in vitro*.

In addition to the role of IL-21 at the DN and DP stages, studies presented in the manuscript demonstrate that IL-21 is important for the survival and proliferation of SP CD8 cells. A significant increase in annexin V expression, as well as reduced BrdU incorporation were observed in the SP CD8 T cell population from the KO compartment of BMC mice, which is consistent with the previously reported role of this cytokine (42, 43). Conversely, the lack of IL-21 signaling does not seem to affect the survival or expansion of the thymic SP CD4 T cell population. Although maturation of IL-21R KO SP CD4 or CD8 T cells appears to be normal, the lower expression of S1P1R on both CD4 and CD8 SP cells from the IL-21R KO compartment as well as their lower ability to migrate to S1P stimulation *in vitro* suggests that these cells have a defect in the ability to migrate to the periphery. As previously reported (12, 42, 43), the role of IL-21 is not limited to the thymic T cell development as our data demonstrate that lack of IL-21 signaling reduced both CD4 and CD8 T cell expansion in the spleen of mixed BMC mice. Furthermore, the role of IL-21 in the abundance of memory CD8 T cells has been reported (45).These observations emphasize the role of IL-21 in homeostatic proliferation and survival of both CD4 and CD8 T cells in the periphery, which is very similar to IL-7 (46).

The data presented in this manuscript define a complex role for IL-21 in thymic T cell development. Even though IL-21 levels in the thymus from the WT animals were significantly lower than IL-7 and IL-15, cytokines which are known to play a role in the thymic development (2, 7), our data demonstrate that lack of IL-21 signaling leads to a suboptimal thymopoiesis. While we do not dispute that IL-7 plays a critical role in thymopoiesis (2, 3), our findings are very important in light of the fact that IL-21R KO mice display normal thymic and splenic cellularity (14) and underline the complex role of IL-21 in thymopoiesis. Normal cellularity observed in IL-21R KO mice could be attributed to the overwhelming predominance of IL-7 in the thymus as compared to IL-21. Furthermore, a potentially unidentified redundant mechanism(s) that may be masking the role of IL-21 cannot be ruled out. The data presented in this manuscript demonstrate a multidimensional role for IL-21 in the development of thymic T cells that may be dependent on IL-7. Based on a published report which showed that IL-7 was significantly increased in the thymus of mice treated with IL-21 (16) and our observation that IL-7 production was decreased in IL-21R KO mice, IL-7 and IL-21 may have a synergistic role in T cell thymic development (47). Our studies raise some important issues related to the thymic T cell development and interactions between IL-7 and IL-21 in this process need to be further studied.

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Key points:

Our study shows that IL-21 plays a pleiotropic role in thymic T cell development.

IL-21 is involved in the differentiation of DN subsets as early as the DN1 stage.

Furthermore, IL-21 seems to be implicated in the emigration of SP CD4 CD8 T cells.

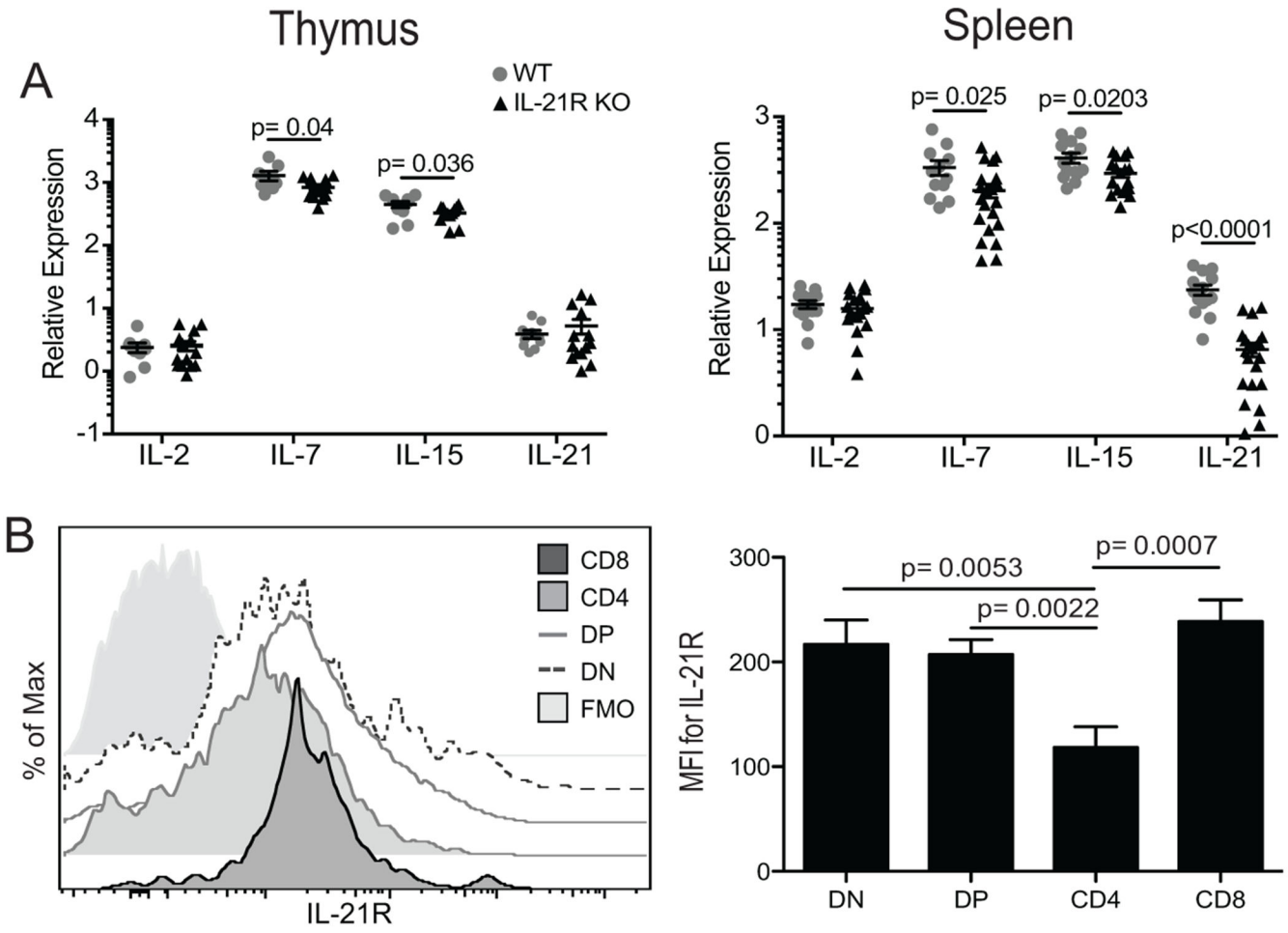

Figure 1: Low IL-21 expression in the thymus

A) Thymus (left) and spleen (right) from WT and IL-21R KO mice were assessed for expression of IL-2, IL-7, IL-15, and IL-21 by real time PCR. Relative expression is presented as ΔCT using β actin as control. Each symbol represents one animal and graphs represent at least three pooled experiments. B) IL-21R expression by thymocytes from WT animals was measured by flow cytometry (n= 3-4 mice/experiment). Graph (right) shows the MFI for IL-21R by DN, DP, SP CD4 and CD8 in the thymus of WT mice. Assay was performed at least twice and data are representative of one experiment.

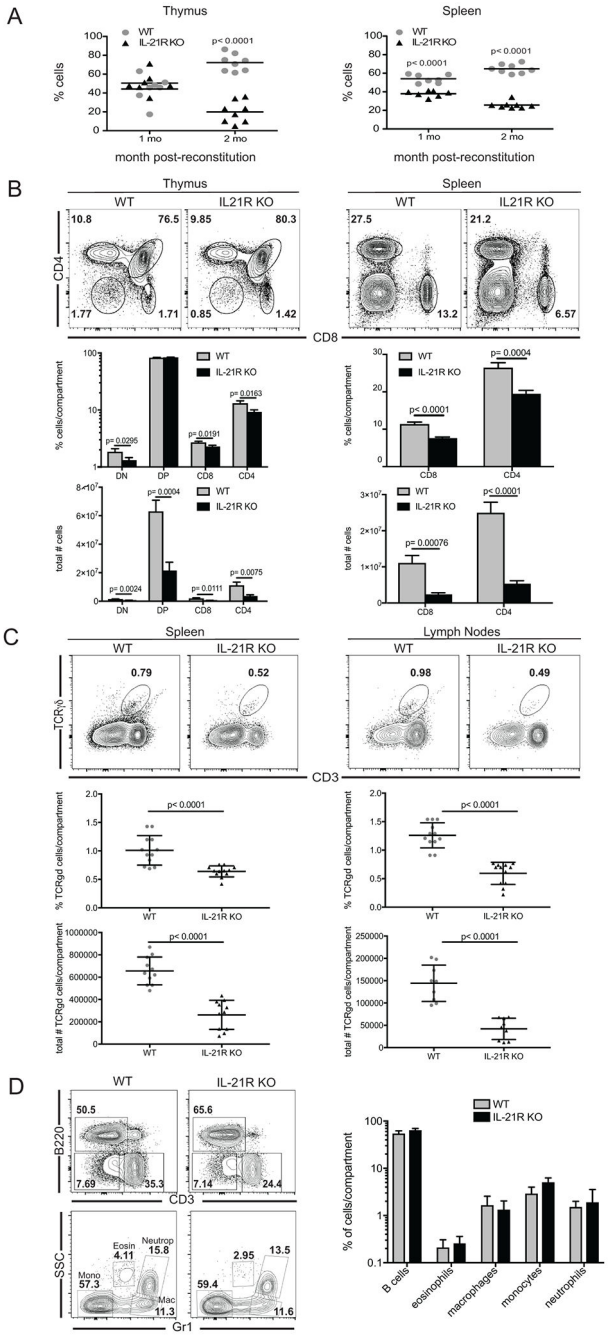

Figure 2: lack of IL-21 signaling affects the thymic T cell development.

Role of IL-21 signaling in thymic T cell development was evaluated using mixed BMC (WT; IL-21R KO, 1: 1). A) Mixed BMC mice (WT/IL-21R KO: 1/1) were assessed for frequency of WT (CD45.1) or IL-21R KO (CD45.2) cells in thymus (left) and spleen (right) 1 and 2 months after reconstitution. B) Frequency and total number of WT or IL-21R KO DN, DP, CD4 and CD8 T cells in the thymus (left) or CD4 and CD8 T cells in spleen (right) in BMC animals (n= 4-5 mice/experiment). C) WT and IL-21R KO TCRγδ cells were analyzed in the spleen (left) and lymph nodes (right) of mixed BMC mice. D) frequency of

B cells ($B220^{+}CD3^{-}$) in WT and IL-21R KO compartments from BMC mice is presented in top panels. In bottom panels, cells gated on $B220^{-}CD3^{-}$ are separated between neutrophils ($SSC^{hi}Gr1^{hi}$), macrophages ($SSC^{lo}Gr1^{hi}$), eosinophils ($SSC^{hi}Gr1^{int}$), and monocytes (also including dendritic cells) ($SSC^{lo}Gr1^{low}$) (n= 3-4 mice/experiment). Graphs represent pooled data from at least 3 experiments with 3-4 mice per experiments (B, C and D).

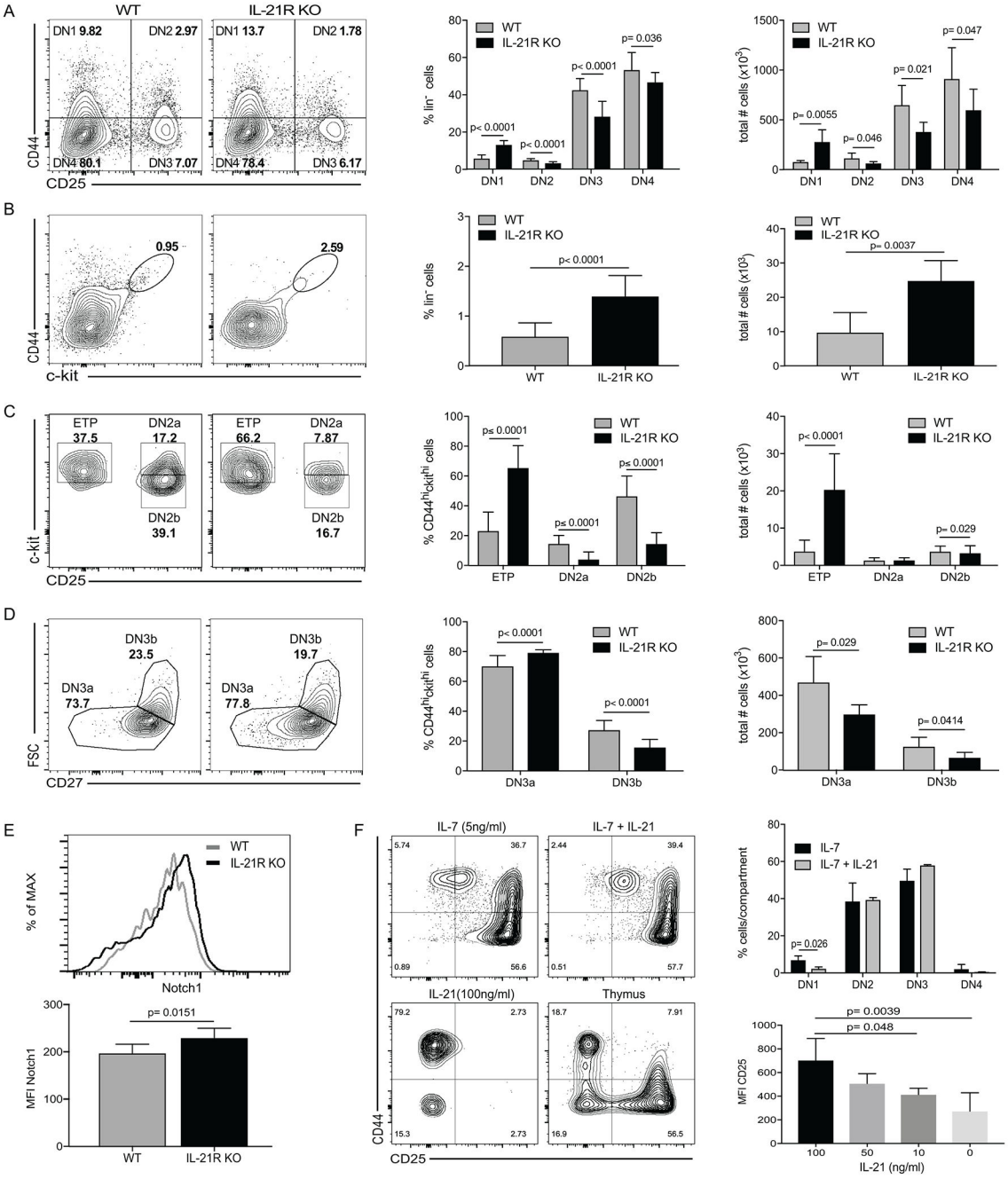

Figure 3: Optimal DN development is dependent on IL-21 signaling.

A) DN1 ($CD44^{hi}CD25^{lo}$), DN2 ($CD44^{hi}CD25^{hi}$), DN3 ($CD44^{lo}CD25^{hi}$), and DN4 ($CD44^{lo}CD25^{lo}$) were gated on WT or IL-21R KO lin^- population from mixed BMC animals at week 8 post-transplant. B) Frequency and number of thymic precursors ($CD44^{hi}c\text{-}Kit^{hi}$) were determined within lin^- population from WT and IL-21R KO compartments from BMC mice. C). Frequencies and total numbers of WT and IL-21R KO ETP ($C\text{-}kit^{hi}CD25^{lo}$), DN2a ($C\text{-}kit^{hi}CD25^{hi}$) and DN2b ($C\text{-}kit^{lo}CD25^{hi}$) subsets were determined after gating on $CD44^{hi}C\text{-}kit^{hi}$ lin^- cells. D) DN3 subset ($CD44^{lo}CD25^{hi}$) was

subdivided into DN3a ($FSC^{lo}CD27^{int}$) and DN3b ($FSC^{hi}CD27^{hi}$). E) WT and IL-21R KO DN populations were analyzed for Notch1 expression by flow cytometry. F) Analysis of DN1 cells cocultured with OP9-DL1 cells for 12 days in presence of IL-7 (5ng/ml), IL-21 (100ng/ml) or IL-7 + IL-21 (5ng/ml and 50ng/ml respectively). Plots are gated on live singlet cells. Lin^- thymocytes were used as a positive control. Bar graphs represent frequencies of DN1,, DN2, DN3, and DN4 after 12 days of coculture in the presence of IL-7 alone or IL-7 + IL-21 and MFI for CD25 (top) on cells recovered from culture treated with different concentrations of IL-21 (100, 50, 10 and 0 ng/ml). Data are representative of at least two experiments (n= 3-4 mice/experiment).

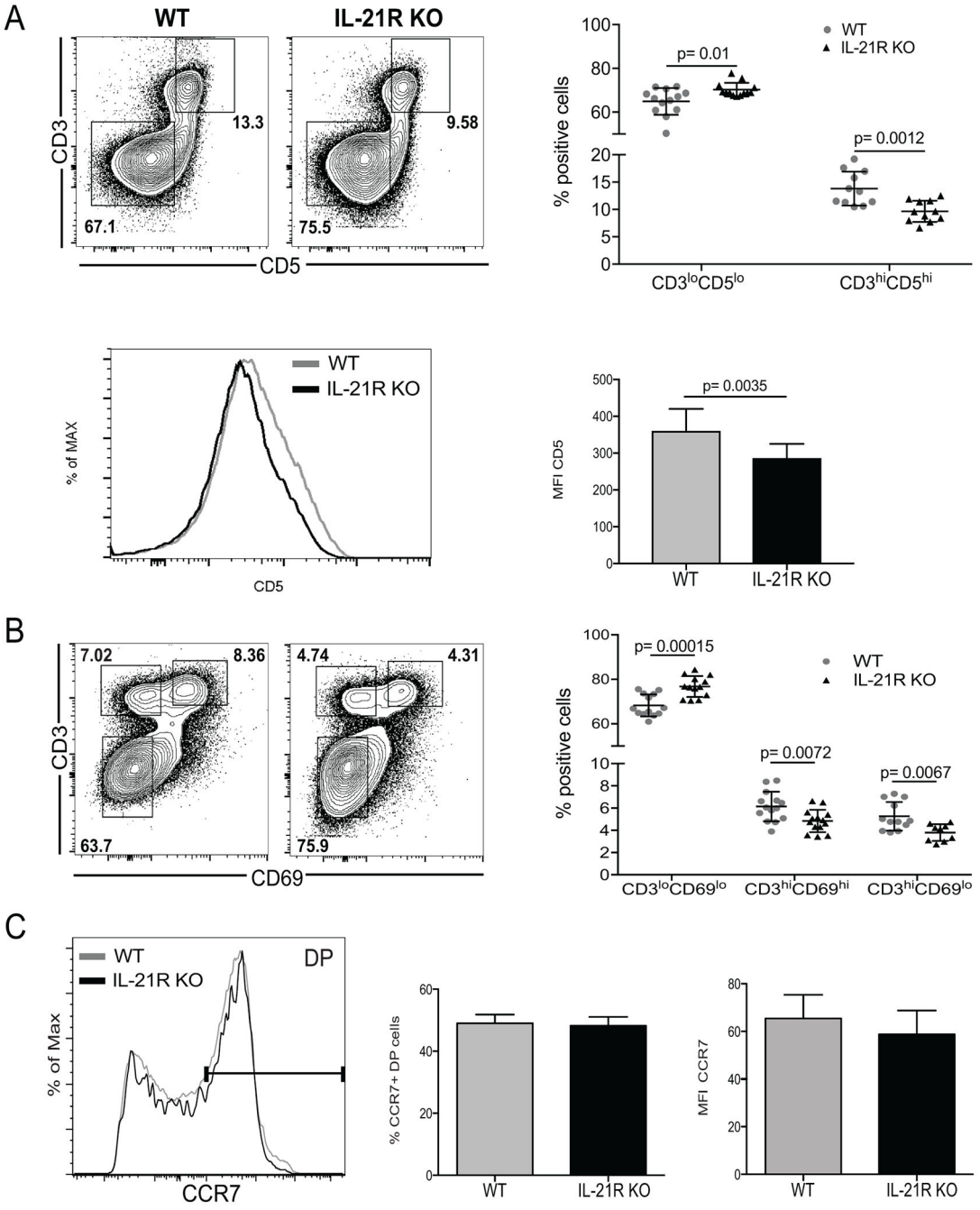

Figure 4: Thymocyte maturation is reduced in absence of IL-21 signaling.

CD3, CD5 and CD69 expression was used to assess the role of IL-21 signaling in the maturation process of total thymic population. A) Mature thymic cells ($CD3^{hi}CD5^{hi}$) were evaluated in WT or IL-21R KO compartments from mixed BMC mice at week 8 post-transplant. Histogram shows CD5 expression by thymocytes from WT and IL-21R KO compartments of BMC animals. B) CD3 and CD69 were used to segregate early post-selection ($CD3^{hi}CD69^{hi}$) and more mature ($CD3^{hi}CD69^{lo}$) populations in WT or IL-21R KO compartments. C) WT and IL-21R KO thymocytes were analyzed for CCR7 expression.

Flow cytometry plots are representative of at least two experiments (n= 3-4 mice/ experiment). Graphs represent pooled data from at least 3 experiments (A and B, right panels).

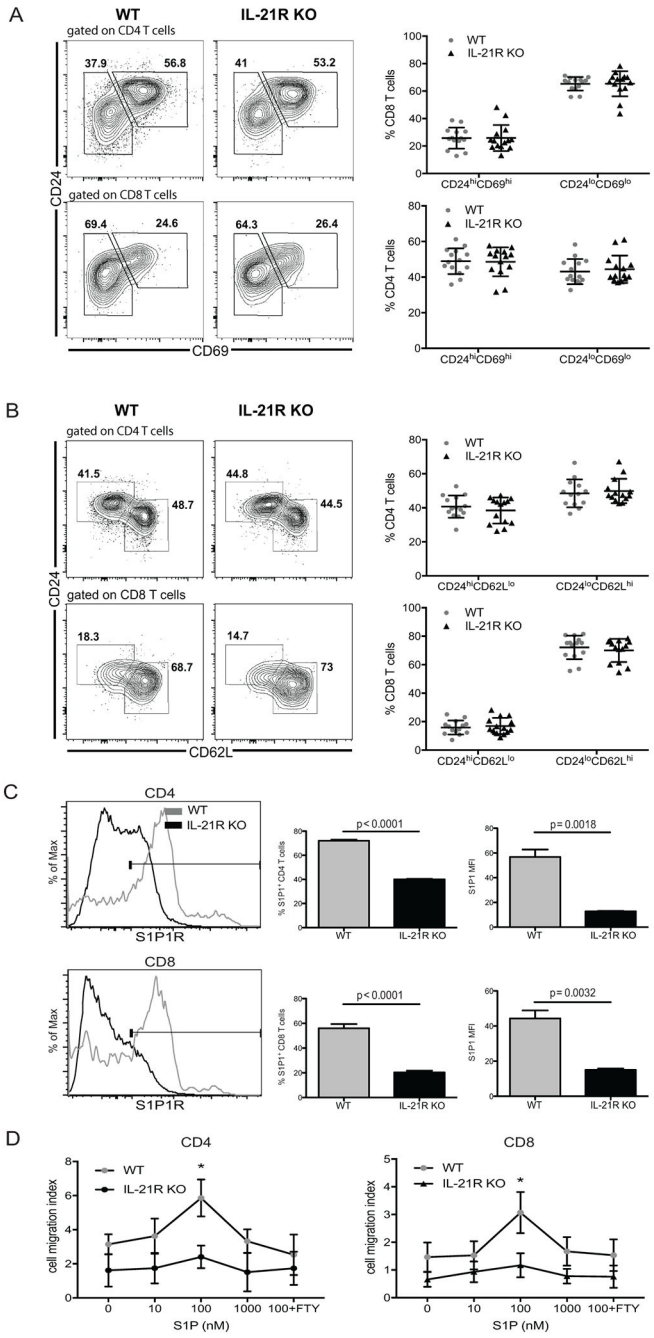

Figure 5: Single positive T cells emigration from the thymus is IL-21 dependent.

The role of IL-21 signaling in maturation and emigration of thymic SP CD4 and CD8 subsets was analyzed in BMC animals. A-B) Mature CD4 and CD8 T cells in thymus from BMC were defined as ($CD24^{lo}CD69^{lo}$) (A) and ($CD24^{lo}CD62L^{hi}$) (B). C) WT and IL-21R KO thymic SP CD4 and CD8 T cells were evaluated for S1P1 expression. Flow cytometry plots are representative of at least two experiments (n= 3-4 mice/experiment). D) Migration of mature CD4 and CD8 ($CD62L^{hi}CD24^{lo}$) from WT and IL-21R KO mice in response to different concentration of S1P (1000, 100, 10, and 0 nM). FTY720 was also used in

conjunction with 100nM of S1P. Statistical significance between WT and IL-21R KO CD4 (p= 0.0016) and CD8 (p= 0.0011) T cells was calculated using a Student's *t*-test. Graphs represent pooled data from at least 3 experiments (A and B, right panels).

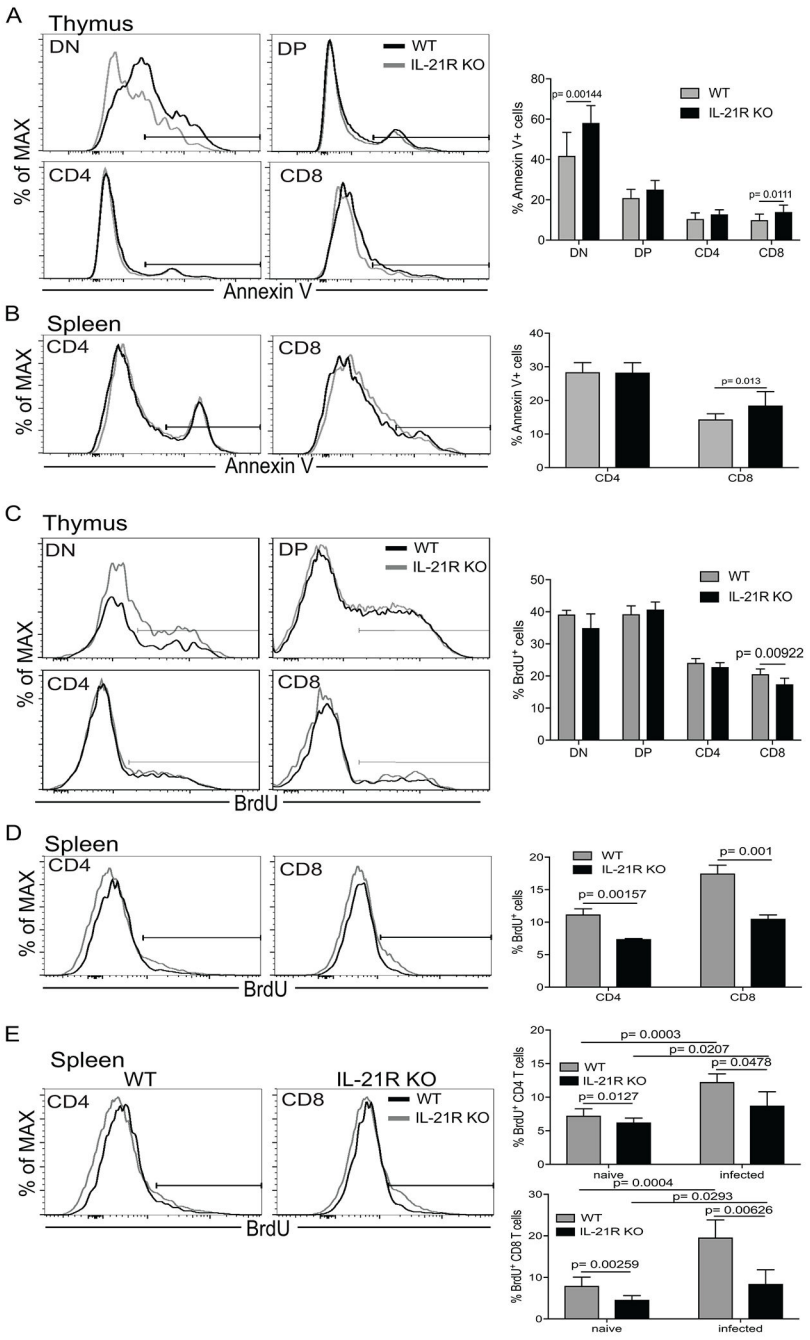

Figure 6: IL-21 plays a role in both survival and expansion of stage specific thymocytes.

Annexin V expression was assessed in the different cell populations from the thymus (DN, DP, SP CD4 and SP CD8) (A) and the spleen (CD4 and CD8 T cells) (B) from mixed BMC mice at week 8 post-reconstitution. BrdU incorporation assay was carried out to measure proliferation of thymic (C) and splenic (D) cell subsets from BMC animals. *In vivo* BrdU treatment was performed for 7 days before animals were sacrificed and intranuclear staining for BrdU was analyzed by flow cytometry. E) BMC animals were infected with *E. cuniculi* and treated with BrdU 7 days later. Expansion of splenic CD4 and CD8 T cells was

measured by BrdU intranuclear staining at day 14 post-infection. Histograms show overlay of WT and IL-21R KO CD4 (left graph) or CD8 (right graph) from infected BMC mice. Assays were performed at least twice and data are representative of one experiment (n= 3-4 mice/experiment).