Université de Montréal

---

# INVESTIGATING THE THYMOPOIESIS-STIMULATING PROPERTY OF INTERLEUKIN-21 IN AGING MICE

---

par
Edouard Al-Chami

Département de Pharmacologie

Faculté de Médecine

Mémoire présenté à la Faculté des Études Supérieures en vue de l'obtention du grade de

Maître ès sciences (M.Sc.) en Pharmacologie

Août 2015

Université de Montréal

Faculté des Études Supérieures

Ce mémoire intitulé :

---

# INVESTIGATING THE THYMOPOIESIS-STIMULATING PROPERTY OF INTERLEUKIN-21 IN AGING MICE

---

a été évalué par un jury composé des personnes suivantes :

Jean-François GAUCHAT , Ph.D.

---

Président-rapporteur

Moutih RAFEI, Ph.D.

---

Directeur de Recherche

Martin G. SIROIS, Ph.D.

---

Membre du jury

## RÉSUMÉ

La vaccination est largement utilisée pour la génération de lymphocytes T spécifiques contre les tumeurs. Malheureusement, cette stratégie n'est pas adaptée aux personnes âgées car leur thymus régresse avec l'âge conduisant ainsi à une baisse dans la production de cellules T et à l'accumulation de cellules immunitaires âgées ayant des défauts liés à leurs stimulations. Comme il a été démontré auparavant que L'IL-21 est capable d'induire des fonctions thymiques, nous avons émis l'hypothèse que l'injection d'IL-21 à des souris âgées stimulera la thymopoïèse. Nos résultats montrent que l'administration de l'IL-21 augmente le nombre absolu de thymocytes chez les souris âgées et augmente la migration de ces cellules vers la périphérie ou ils contribuent à la diversité du TCR. De plus les cellules T en périphérie expriment un niveau plus élevé de miR181-a, et par conséquent moins de phosphatase comme SHP2, DUSP5/6 qui inhibent le TCR. En vaccinant des souris âgées avec le peptide Trp2, les souris traitées avec l'IL-21 montrent un retard dans la croissance des cellules B16 tumorales. Cette étude montre que l'IL-21 pourrait être utilisé comme stratégie pour le rétablissement du systeme immunitaire chez les personnes âgées.



**Mots clés:** Involution thymique; Interleukine-21; Cellules T; Signalisation; miR-181a; Vaccin.

## ABSTRACT

Vaccines have been largely sought for the generation of protective tumor-specific T cells. Unfortunately however, this strategy is not suitable for the elderly as their thymus regresses with age leading to a decline in T-cell production and accumulation of aged lymphocytes endowed with stimulation-related defects. Interleukin (IL)-21 has been shown to display thymo-stimulatory properties. Therefore, we hypothesized that IL-21 administration to ageing host may boost T-cell production and restore a competent peripheral T-cell compartment. Our study shows that IL-21 injection to aged mice lead to an increase in the thymocytes absolute number and an increase in thymocytes egress to the periphery where they enhance the T-cell receptor (TCR) diversity. Furthermore T-cell in the periphery express higher level of miR-181a and thus less TCR-inhibiting phosphatases SHP-2 and DUSP5/6 enable them to be more responsive upon stimulation. Consequently, aged rIL-21-treated mice vaccinated using a tyrosinase-related protein 2 (Trp2)-derived peptide exhibited a substantial delay in B16 tumor growth and improved survival. The results of this study highlight the immunorestorative function of rIL-21 paving its use as a strategy for the re-establishment of effective immunity in the elderly.



**Key words:** Thymic Involution; Interleukin-21; T cells; Signaling; miR-181a; Tumor Vaccine

## ACKNOWLEDGEMENTS

First of all, I would like to thank my research supervisor Dr. Moutih Rafei for giving me the opportunity to work in his laboratory. The training I acquired during the last two years was full of challenges, which turned into victories thanks to Dr. Rafei's patience, intelligence and guidance. I was very lucky to work under his supervision. He invested so much in training me and I hope I was at his level of expectations. He helped me writing my scholarship applications, corrected my Power Point presentations and even assisted me in some of my graduate courses. Dr. Rafei is a young investigator full of energy and I wish him the best of luck for the rest of his career. I learned a lot from him and I hope he learned something from me too.

I would also like to thank Dr. Aurélie Tormo for her patience and help. Aurélie was always around for help. Her long expertise in research made my master degree much easier. Thank you for showing and teaching me all the techniques that I used and all the small tricks that allowed me to save time. Many thanks for correcting my terrible French in all of my applications and e-mails. You are such a wonderful lab manager, wish you all the best!

I would also like to thank Dr. Jean-Francois Gauchat. Besides being an expert in Immunology and Molecular Biology, he helped me a lot through his wise feedbacks. What fascinates me the most in Dr. Gauchat is his humbleness. Whenever you cross him in the hallway, he will always smile and greet you. Thank you for charging my days with positive energy! May the force be always with you!

***"Impossible is a word to be found only in the dictionary of fools."***

- Napoleon Bonaparte

# PREFACE

Almost 50% of cancer cases are diagnosed in patients aged 60 years and older. As more than 25% of the total U.S. population will reach the age of 60 by 2050, the number of patients suffering from cancer is expected to rise. Since living standards and overall healthcare are improving worldwide, a higher life expectancy requires the adoption of novel strategies aimed at keeping the aging population healthy. Although most cancer types can be removed by surgery, followed by radiation and/or chemotherapy, these multimodality therapies are ineffective against spread metastases. Cancer vaccination represents therefore an appealing alternative due to its limited toxicity and ability to stimulate the host's own immune system to seek and destroy metastatic cancer cells. Unfortunately however, vaccines are less effective at older age due to impaired T-cell responsiveness due to immunosenescence; a degeneration of the immune system caused by thymic atrophy. As a result, the output of newly developed naïve T cells endowed with the capacity of effectively responding to new antigenic challenges dramatically decreases pocking major holes in patients' immunocompetency. Thus, there is an urgent need for the development or improvement of immunotherapies to suit the elderly population. The study described herein will highlight the utility and potency of interleukin (**IL**)-21 at stimulating *de novo* thymopoiesis to rejuvenate the immune system of aged subjects. Optimising and adopting such concept will lead to innovative IL-21-based strategies directed against two key elderly-related health problems: i) providing superior responsiveness to cancer vaccines, and ii) reducing the emergence and/or cancer relapse in this vulnerable population. Therefore, the favourable outcome of this study will have significant ramifications on the future use of IL-21 in the context of preventive/therapeutic cancer vaccination measures aimed at enhancing anti-tumoral immunity of the aging population.

## TABLE OF CONTENTS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| **AP-1**: | Activator Protein-1 |
| **AR**: | Androgen Receptor |
| **Bcl6**: | B-Cell Lymphoma 6 Protein |
| **Bim**: | Bcl-2-Like Protein 11 |
| **BM**: | Bone Marrow |
| **BMT**: | Bone Marrow Transplantation |
| **CCL**: | C-C Motif Ligand |
| **CCR**: | C-C Motif Receptor |
| **CD**: | Cluster of Differentiation |
| **CLP**: | Common Lymphoid Progenitor |
| **cTECs**: | Cortical Thymic Epithelial Cells |
| **CTP:** | Circulating Bone-Marrow Derived T-Lineage Progenitor |
| **DC**: | Dendritic Cells |
| **DEX**: | Dexamethasone |
| **DL4**: | Delta-Like 4 |
| **DN**: | Double-Negative |
| **DP**: | Double-Positive |
| **DUSP5/6**: | Dual Specificity Phosphatase 5/6 |
| **ELISA**: | Enzyme-Linked Immunosorbent Assay |
| **ERK**: | Extracellular Signal-Regulated Kinases |
| **ETPs**: | Early Thymic Progenitors |

**FOXN1**: Forkhead Box N1

**FOXP3**: Forkhead Box P3

**FSH:** Follicle Stimulating Hormone

**GATA3**: Trans-Acting T-Cell-Specific Transcription Factor

**GFP**: Green Fluorescent Protein

**GH:** Growth Hormone

**GM-CSF**: Granulocyte Macrophage-Colony Stimulating Factor

**GRL**: Ghrelin

**GRLR**: Ghrelin Receptor

**GVHD:** Graft Versus Host Disease

**Gzm**: Granzyme

**HSC**: Hematopoietic Stem Cells

**IFNγ**: Interferon-Gamma

**IL**: Interleukin

**IL-21R**: Interleukin-21 Receptor

**IP**: Intraperitoneal

**KGF**: Keratinocyte Growth Factor

**KGFR**: Keratinocyte Growth Factor Receptor

**Lck**: Lymphocyte-Specific Protein Tyrosine Kinase

**LH:** Luteinizing Hormone

**LHRH**: Luteinizing Hormone Releasing Hormone

**LPS**: Lipopolysaccharide

**LSK**: $Lin^{-}Sca1^{+}c\text{-}kit^{+}$

**LT-HSC**: Long Term-HSC

**MFI**: Mean Fluorescent Intensity

**MPP**: Multi-Potent Progenitor

**mTECs**: Medullary Thymic Epithelial Cells

**NFAT**: Nuclear Factor of Activated T-cells

**NK**: Natural Killer

**OVA**: Ovalbumin

**PBS**: Phosphate Buffer Saline

**Prdm1**: PRD Zinc Finger Protein 1

**PTPN22**: Protein Tyrosine Phosphatase Non Receptor Type 22

**qPCR**: Quantitative Polymerase Chain Reaction

**RAG**: Recombination-Activating Genes

**RNA**: Ribonucleic Acid

**RORC**: RAR-Related Orphan Receptor C

**RORγt**: RAR-Related Orphan Receptor Gamma

**RTE**: Recent Thymic Emigrant

**SLO**: Secondary Lymphoid Organ

**SP**: Single-Positive

**STAT**: Signal Transducer and Activator of Transcription

**ST-HSC**: Short Term-HSC

**SOCS**: Suppressor of Cytokine Signaling

**SSA:** Sex Steroid Ablation

**T-bet**: T-box transcription factor

**TCR**: T-Cell Receptor

**TES**: Thymic Epithelial Space

**TFH**: T-Follicular Helper

**TREC**: TCR Excision Circles

**TRP-2**: Tyrosinase-Related Protein 2

**TSP**: Thymic Seeding Progenitors

**WT**: Wild-Type

**XSCID**: X-linked Severe Combined Immunodeficiency

**ZAP-70**: Zeta-Chain-Associated Protein kinase 70

## LIST OF FIGURES

# CHAPTRE 1

## *GENERAL INTRODUCTION*

## 1.0 - Aging and Thymopoiesis

One of the most important attribute of adaptive immunity is its capacity to discriminate between self and non-self-antigens (1). In order to achieve that, its main components, B and T lymphocytes, must undergo restricted processes during their development (2). In jawed vertebrates including humans, B and T cells develop at distinct anatomical sites, the former in the bone marrow (**BM**) or fetal liver and the latter in the thymus (2). From an evolutionary point of view, we can assume that the thymus emerged as a dedicated environment to facilitate and support the development of self-tolerant T cells expressing a diverse repertoire of T-cell receptor (**TCR**) (3). Failure to build or maintain a proper thymus can lead to defects ranging from immunodeficiency to autoimmunity (4).

The main thymus-associated limitation is the progressive decrease of its function with age; a phenomenon known as thymic involution (5). Age-associated thymic involution occurs in all species, indicating that this process is evolutionary ancient and conserved. It is characterized by a reduction in mass, cellularity as well as loss of normal architecture consequently leading to a decrease in T-cell development (thymopoiesis) (6). As a result, diminished production of naïve T cells and attrition of TCR repertoire in aging hosts is amenable to increased susceptibility to infectious diseases, cancers and autoimmunity (6). From a general perspective, thymic involution can be considered as a natural leading global health problem. Thus, a tremendous amount of effort is put to prevent or reverse age-associated thymic involution as a mean to ensure the well-being of the aging society.

## 1.1 - Thymus structure

The thymus (**FIGURE 1**) consists of two distinct lobes connected by connective tissues and surrounded by capsular tissues (7). Histologically, each lobe can be subdivided into four major compartments each having a distinct role in thymopoiesis. The four compartments include the subcapsular zone, the cortex, the medulla, and the cortico-medullary junction (4). The subcapsular zone contains mainly cortical thymic epithelial cells (**cTECs**), whereas the cortex contains a mix of cTECs, fibroblasts and macrophages. The medulla is comprised of medullary thymic epithelial cells (**mTECs**) and dendritic cells (**DC**). The cortico-medullary junction is the vascular region where thymic arteries enter the organ. The stroma is considered as the non-hematopoietic component of the thymus and contains keratin positive and negative cells. Keratin positive cells represent TECs and it is divided into keratin5$^-$ keratin8$^+$ (K5$^-$k8$^+$ for cTECs) and keratin5$^+$ keratin8$^-$ (k5$^+$k8$^-$ for mTECs) cells. Keratin negative cells are a mixture of fibroblasts, non-fibroblastics mesenchymal cells and endothelial cells (4). The thymus stroma is essential to the regulation of T-cell development and selection. For example, TECs secrete chemokine and growth factors and express cell surface molecules all involved in supporting migration and development of progenitor cells (8). In addition, TECs present distinct peptide repertoires essential for central tolerance (8). During thymic involution, the thymus stroma degenerates and is subsequently replaced by adipose tissue, which negatively interferes with thymopoiesis.

## 1.2 - Intrathymic T-cell development

Thymopoiesis involves several differentiation and proliferation events during which cross-talks between stromal and lymphoid cells take place (10) (**FIGURE 1**). Lymphoid progenitor cells

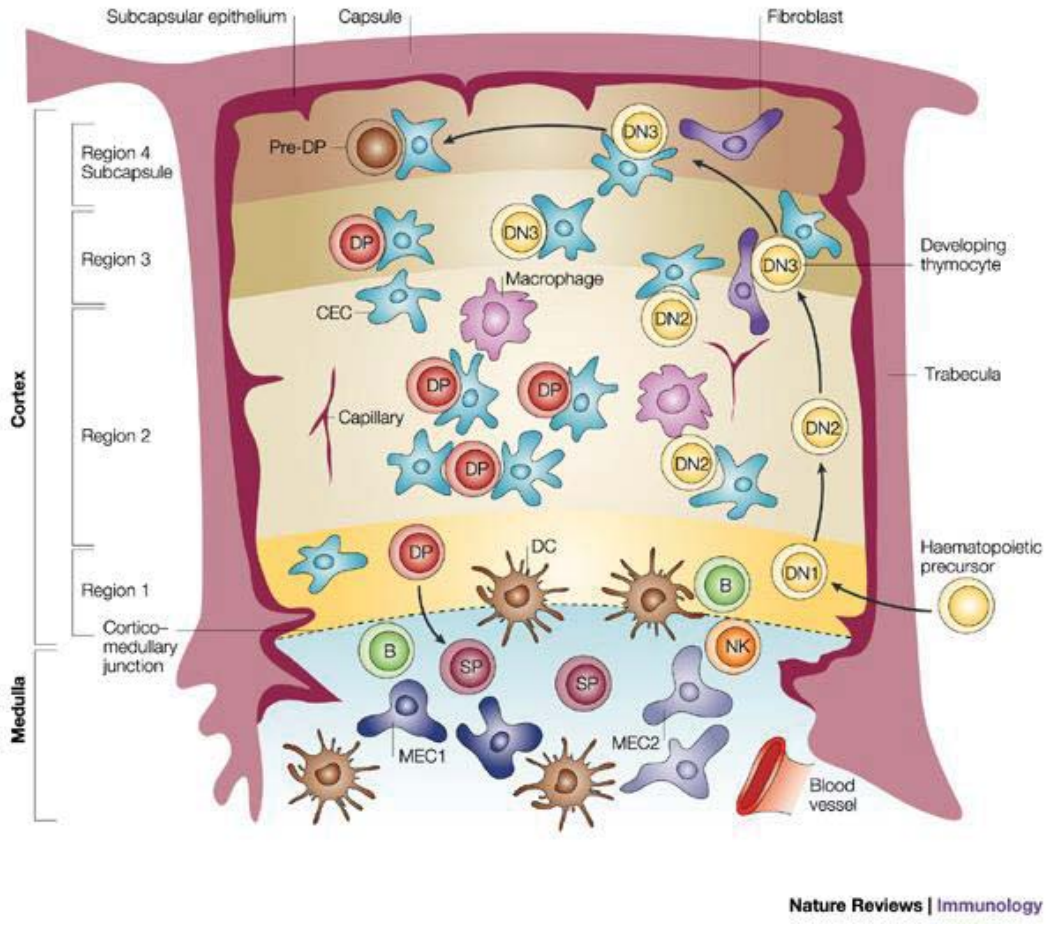



**FIGURE 1: THYMIC STRUCTURE AND T-CELL DEVELOPMENT.**

A simplified overview of the thymus defined regions and stages of T-cell development. (**Blackburn CC *et al*. Nature Reviews Immunology 4, 278-289 (2004**) (9).

migrate from the BM, enter the thymus via the cortico-medullary junction, and undergo four stages of maturation as they pass from the subcapsular zone through the cortex and to the medulla before reaching finally peripheral circulation as immature naïve T cells (7). For that to happen, T-cell migration to and through the thymus is guided by chemokines produced by thymic stromal cells in distinct regions or microenvironments, and developing thymocytes seem to find their way by sequentially expressing different chemokine receptors specific for the transition between each stage of maturation (11). Developing thymocytes are sub-divided into three major groups based on the expression of CD4 and CD8 on their cell surface. They start as double-negative (**DN**) cells that do not express CD4 or CD8 and after different maturation processes they become double positive (**DP**) cells expressing both CD4 and CD8. They finally become single-positive (**SP**) T cells expressing either the CD4 or CD8 co-receptor (11). DN thymocytes can be further sub-divided into four sub-populations according to their expression of CD44 and CD25: DN1 ($CD44^{+}CD25^{-}$), DN2 ($CD44^{+}CD25^{+}$), DN3 ($CD44^{-}CD25^{+}$), and DN4 ($CD44^{-}CD25^{-}$). The first step in T-cell development is the migration of lymphoid progenitor cells or thymic seeding progenitors (**TSP**) from the BM to the thymus. This step is controlled by the adhesive interaction between platelet P-selectin glycoprotein ligand 1, which is expressed by circulating TSP, and P-selectin found on the surface of thymic endothelium. They enter the thymus via the vasculature at the cortico-medullary region and become early thymic progenitors (**ETPs**) (the most important sub-population of the DN1 fraction). ETPs still retain the potential to give rise to DC, natural killer (**NK**), and macrophages, but they lose their multipotency as they differentiate and become restricted to the T-cell lineage (12). The first checkpoint in the developmental process of T cells is the T lineage commitment at the DN1/DN2 stages. In order to become committed to the T-cell lineage, DN1 cells interact with the delta-like 4 (**DL4**) ligand

expressed on cTECs through their Notch1 receptor (11). In addition, cTECs secrete interleukin (**IL**)-7 which supports their differentiation process. DN2 differentiate to DN3 in the subcapsular zone and during this transition the αβ versus γδ T cells fate is specified. The DN3 cells follow another checkpoint known as the β-selection where only DN3 who have successfully rearranged the β-chain of the TCR survive by receiving a signal through a functional pre-TCR complex (11). Thymocytes that have been selected proceed to the DN4 stage, initiate CD4 and CD8 expression and become DP thymocytes in the cortex. At that point, DP cells initiate the TCRα gene rearrangement resulting in the expression of TCRαβ complexes. DP cells in the cortex will then undergo positive selection where only thymocytes with a functional TCR capable of recognizing self-peptide-MHC complexes presented by cTECs receive a survival signal and differentiate into SP cells (13). Surviving DPs will then express the C-C chemokine receptor type (**CCR**) 7, which guides them to the medulla in response to CCR7 ligands (C-C motif ligand (**CCL**)19 and CCL21). In the medulla, mTECs and DCs will express and present tissue-specific antigens to SP cells to negatively select any TCR with self-reactive potential in order to avoid generating reactive T cells capable of causing autoimmunity (13). SP thymocytes will spend ~12 days in the medulla whereby they will express the sphingosine-1-phosphate receptor 1. At that time point, CD4 or CD8 T cells egress from the thymus and continue their maturation in the periphery for an additional period of three weeks (13).

### 1.3 - Thymic Involution from an Evolutionary Perspective

Thymic involution is characterized by the expansion of perivascular space due to adipocytes accumulation with age shifting the ratio of thymic epithelial space (**TES**) to perivascular space (5). Consequently, the TES represents less than 10% of total thymic tissue by the age of 70 (5).

Such thymic regression results in constriction of peripheral T-cell diversity, alterations in their phenotype and function, and corrosion of telomeres causing replicative senescence (6). Age-associated thymic involution has been suggested to occur in all species, indicating that this process is evolutionary ancient and conserved (14). If so, are there any benefits to thymic involution? The human life span has extended drastically over the last centuries due to decline in infectious disease-caused mortality owing to improved sanitation, medical care as well as development of vaccines and antibiotics (15). This means that the immune system, which was once "designed to serve for almost 40 years" has to provide continuous protection for the decades to come (16). Of all evolutionary hypotheses, the "disposable soma theory", which suggests that the body must budget its available amount of energy between locomotion, thermogenesis, growth, reproduction and repair appears to be highly compatible with thymic involution as the thymus is an "energy expensive organ" due to the fact that more than 90% of all developed thymocytes die daily (17). Under such context, thymic involution is divided in two main stages and may be regulated by different mechanisms (18). The first stage occurs early in life mostly at 6 weeks of age in mice and at one year after birth in humans (19). This early thymic involution is believed to happen as newborns have essentially an "empty" peripheral immune compartment (20). Thus, the thymus increases its T-cell output capacity for the purpose of populating the peripheral immune-based compartment (18). Once this task fulfilled, thymic size and output are reduced (18). The second thymic involution wave is gradual throughout life and starts after puberty (24). Briefly, the TES begins to involute at a rate of 3% per year until middle age (35-45 years) and continues afterwards to decrease at a rate of 1% per year throughout the rest of life (5). Little is known about the precise mechanisms that lead to the second stage of thymic involution, but a substantial amount of evidence suggests that both extrinsic and intrinsic factors

may be involved (5). In fact, acute thymic involution caused by extrinsic stimuli continuously occur especially when energy and resources are scarce in certain conditions including starvation, malnutrition and pregnancy (18). In support of this notion, the exogenous administration of the energy intake regulating hormone leptin has been shown to prevent thymic atrophy (21).

## 1.4 - Factors Causing Thymic Involution

It is well established nowadays that thymic involution is based on two essential factors: i) a defect in the survival/proliferation/differentiation abilities of the pre-thymic hematopoietic progenitor pool, and ii) the precious loss of TECs (22). In support of the former point, several studies have shown that transplanting BM derived from aged mice into irradiated recipient younger hosts is less efficient at reconstituting the hematopoietic system (23). This is explained by the fact that aging hematopoietic stem cells (**HSCs**) are biased towards myeloid differentiation (24). Interestingly however, when young BM is transferred to aged recipient mice, a decrease in thymic reconstitution was also observed indicating that alterations in thymic microenvironment can also negatively impact thymopoiesis (25). Such alterations can be caused by stress factors capable of disrupting the homeostatic balance of the immune system (**FIGURE 2**). For example, physiological stress such as malnutrition, infections, irradiation, and pregnancy trigger transient but reversible acute thymic involution (6). This is different from chronic age-associated thymic involution in that acute atrophy is characterized by enhanced death of thymocytes followed by a recovery phase after the insult has been removed (18). So far, no approved treatments are available to protect against acute and/or chronic thymic atrophy, thereby leaving the immune system compromised under these conditions.

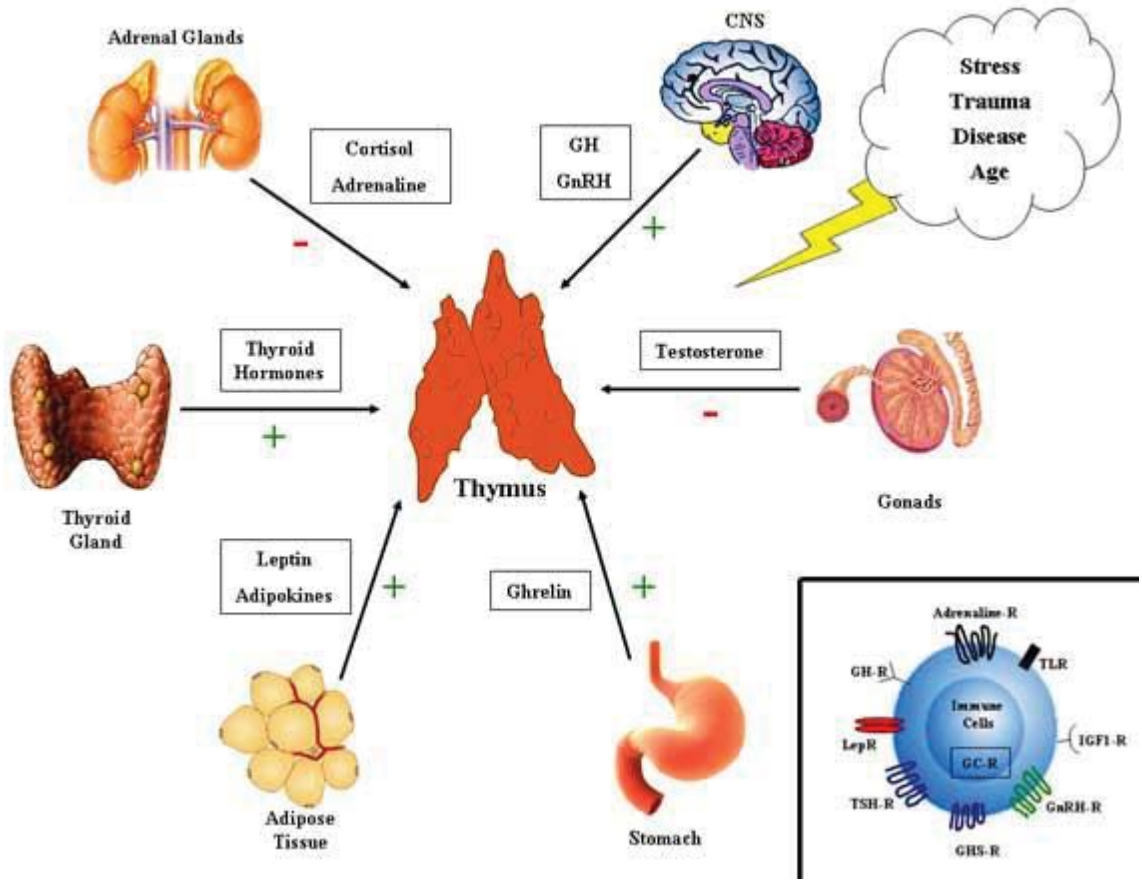



**FIGURE 2: EXTRINSIC FACTORS AFFECTING THYMUS HOMEOSTASIS.**

Besides age-related chronic involution, several extrinsic factors can cause acute thymic atrophy including viral or bacterial infections, strong inflammation, malnutrition or pre-conditioning treatments such as chemo- or radiotherapies given prior to some medical interventions. GH, Growth Hormone. (**Patel K. *et al*. Biology Reports 2009)** (25).

## 1.5 - Experimental Strategies to Reconstitute the Thymus

There is a growing body of evidence that the thymic tissue is plastic and that the involution process might be therapeutically halted or reversed. Therefore, understanding the processes triggering declines in thymic function during aging would help in the development of strategies that can reverse thymic atrophy and improve the overall outcome of immunocompromised patients. As restoration of immune competence is critically dependent on residual thymic function, most attempts to regenerate the thymus involve factors targeting the stromal compartment. Of all strategies developed so far, we will only provide a brief overview on the most promising ones.

### 1.5.1 - Sex Steroid Ablation (SSA)

The decline in size and function of the thymus is more pronounced from the onset of puberty, which fits the increase in circulating sex steroids at that time (27). Therefore, increased sex steroids secretion during puberty has been proposed to contribute to the process of thymic involution. Several studies have validated this hypothesis either by administering sex steroids (androgens or estrogens) in young pre-pubertal mice to promote thymic involution or through their neutralization as a mean to inhibit/reverse thymic involution (27). Mechanistically, steroid hormones mediate their biological effects by interacting with their cognate receptors. Androgen and estrogen receptors are expressed in male and female thymi in both the hematopoietic and stromal compartments (28). In most instances, after binding its respective sex steroid, the receptor translocate to the nucleus where it directly mediates changes in the expression of target genes. As a result, male sex steroids induce $CD4^{+}CD8^{+}$ DP thymocytes apoptosis though the up-regulation of tumor-necrosis factor-α, (29) while estrogens trigger thymic atrophy by eliminating

ETPs and inhibiting the proliferation of thymocytes at the β-selection stage (30). Based on these observations, SSA was proposed as a rational strategy to boost thymic function and promote thymic rejuvenation in young and adult subjects (**FIGURE 3**). Although surgical castration displays beneficial effects on thymic cellularity, architecture, organization, and enhances thymopoiesis in both young and adult animals, its therapeutic translation cannot be ensured due to its irreversible effects and ethical considerations (except for prostate cancer patients). However, a transient and reversible approach using compounds targeting the upstream signaling events such as the luteinizing hormone releasing hormone (**LHRH**) or directly blocking sex steroid receptors originally developed for prostate and breast cancer patients can be used (31). For instance, mice treated with the LHRH-agonist Lupron (aka ASC-J9) showed an increase in the number of lymphoid and myeloid progenitors in the BM and increased thymic and splenic recovery after BM transplantation (**BMT**) (32). In addition, clinical trials of SSA have shown that the use of LHRH-agonist in a small cohort of prostate cancer patients between 60 and 77 years enhances thymic function (increased levels of naïve $CD4^+$ and $CD8^+$ T cells and NK counts 4 months post-treatment) (24). Analysis of thymic function by measuring recent thymic emigrants by TCR excision circles (**TREC**) revealed that 8 of the 10 patients showed an increase in the total $TREC^+$ cells/ml of blood compared to placebo (24). Similarly, a non-randomized pilot study involving patients undergoing BMT showed a significant increase in naïve $TREC^+$ T cells with the use of the LHRH-Agonist Goserelin (33). These clinical studies clearly demonstrate that SSA represents a valid strategy to enhance thymic function, not just in immunocompromised patients, but also during aging. However, the systemic effects of SSA in the long term run is not yet clear nor are the potential systemic side effects. Thus, more in depth investigations are required to assess the safety of SSA-based compounds.

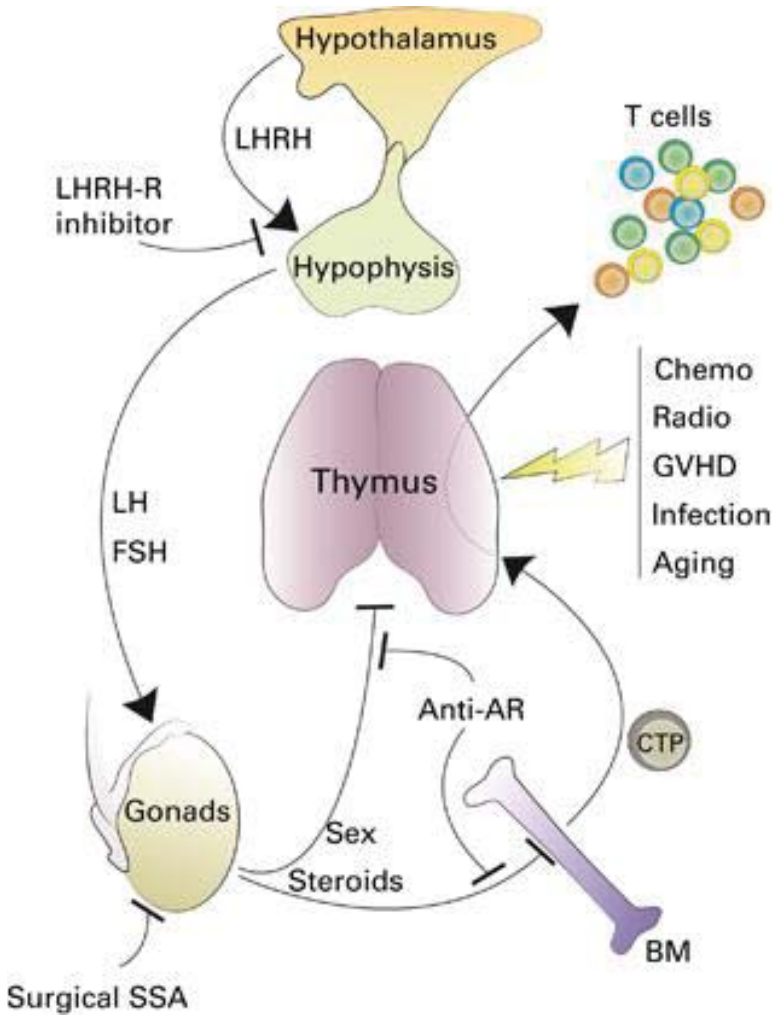



**FIGURE 3: OVERVIEW OF THE HYPOTHALAMIC–PITUITARY–GONADAL AXIS AND ITS IMPLICATION IN REGULATING THYMIC FUNCTION.**

SSA using LHRH-R inhibitors or anti-androgen receptors (**AR**), blocks the negative effects of sex steroids on BM and the thymus and promotes their rejuvenation in steady-state conditions as well as following immune insults. LHRH, Luteinizing Hormone Releasing Hormone; LH, Luteinizing Hormone; FSH, Follicle Stimulating Hormone; GVHD, Graft Versus Host Disease;

CTP, Circulating Bone Marrow-Derived T-lineage Progenitor. (**Velardi E. *et al*. Bone Marrow Transplant 2015. 50, S77–S81)** (32).

### 1.5.2 - Keratinocyte Growth Factor (KGF): the Stalwart of Thymic Epithelium Protection

In the thymus, KGF is produced by thymic stromal cells and thymocytes, but its receptor (**KGFR**) is only expressed by TECs (35). KGFR-deficient mice have arrested thymic development (35). In contrast, an increase in the proportion of naïve T cells is observed following KGF administration to mice undergoing allogeneic BMT (36). Because KGFR is expressed by many organs targeted by alloreactive T cells, several research groups studied the effects of KGF in the setting of acute graft-versus-host disease (**GVHD**). Administration of KGF under such context led to: i) facilitated allo-engraftment, ii) alleviated GVHD, iii) protected and repaired epithelial cell in the gut mucosa, iv) reduced inflammatory cytokine release, and v) diminished allogeneic T-cell responses (36). Furthermore, KGF was recently reported to inhibit *Ink4a expression;* a tumor-suppressor gene repressing proliferation of T-cell progenitors (37). This was unexpected as KGFR is exclusively expressed by TECs. To explain this contradictory finding, Berent-Maoz *et al*. suggested that KGF triggers TECs to secrete soluble factors, which act on T-cell progenitors and down-regulate *Ink4a* expression (37). As a result, the proliferative potential of ETP and DNs is increased leading to enhanced T-cell progenitors output. KGF may therefore protect TECs while stimulating thymopoiesis to levels sufficient to boost the peripheral pool of naïve T cells and improve impaired immune functions. To validate these observations clinically, two separate phase I/II trials were conducted on patients undergoing allogeneic BMT (38). Although treated patients displayed ameliorated mucositis, an inflammation and ulceration of the mucous membranes lining the digestive tract, no significant improvements were observed

with respect to the incidence and severity of acute GVHD, T-lymphopoiesis, infections, overall survival, or cancer relapse rates (39). Altogether, KGF ameliorates mucotoxicity following allogeneic BMT without any favourable impact on immune recovery in patients.

### 1.5.3 - Ghrelin (GRL)

GRL is a 28 amino acid mature polypeptide released from the stomach into the circulation where it stimulates the feeding center of the hypothalamus to induce hunger (41). The GRL receptor (**GRLR**), on the other hand, is highly expressed by the pituitary gland, the central nervous system and on various immune cells (40). GRL administration has been shown to inhibit adipogenesis and inflammation in various preclinical models whereas its deficiency leads to reduced thymopoiesis and increased thymic adiposity (40). As thymic involution is naturally accompanied by adipocytes accumulation and increased production of pro-inflammatory cytokines, a role for GRL in reversing age-related thymic involution was proposed (40). To this end, Dixit *et al*. demonstrated that GRL administration to 14-22 months aged mice resulted in: i) increased thymic size and cellularity, ii) loss of thymic adipocytes, iii) increased number of both c- and mTECs, ETPs, recent thymic emigrants (**RTEs**), and finally iv) improved TCR diversity (40). Although GRL shows impressive pleiotropic thymopoietic-stimulating effects, its use as a potential therapy may be limited by the progressive loss of thymic GRLR expression with aging (40).

### 1.5.4 - IL-7: The First Thymopoietin

Initially discovered in 1988 as a murine B-cell precursor growth factor, IL-7 was quickly found to enhance: i) expansion of naïve peripheral CD4 and CD8 SP T cells, ii) antiviral/antitumour

activity of cytotoxic T cells, and iii) survival/proliferation of CD8 memory T cells (42). Based on these properties, IL-7 was extensively tested as an immunotherapeutic in the context of infections, cancer therapies and for T-cell reconstitution following BMT (42). Although these preclinical studies defined IL-7 as the cornerstone of T-lymphopoiesis, studies conducted in higher species contradicted this notion. For example, an autologous $CD34^+$ cell transplantation study aimed at reconstituting peripheral T cells in non-human primates was inconsistent with observations made in rodent models. IL-7 administration to transplanted baboons had profound proliferative effects on peripheral T cells with marginal *de novo* thymopoiesis (43). Furthermore, two IL-7 phase I clinical trials conducted on cancer patients failed to provide evidence for thymopoiesis. In the first trial, IL-7 administration to 16 subjects with refractory malignancy (age ranging from 20-71 years old) led to preferential expansion of RTEs and memory T cells (44). The second trial, on the other hand, investigated the impact of IL-7 administration on gp100/MART-1-based vaccination of 12 patients aged 20-67 with metastatic melanoma/sarcoma. In addition to the absence of enhanced anti-tumoral effects, no significant increase in naïve T-cell frequency was observed (45). Even though the lack of evidence for IL-7 in triggering thymopoiesis in higher species is yet to be clarified, IL-7 may still be clinically relevant as a potent adjuvant to stimulate T-cell effector functions.

### 1.5.5 – Exploiting the IL-22/IL-22R Axis to Rebuild the Thymus

Since its discovery in 2000, knowledge of IL-22 biology has evolved rapidly. Part of the IL-10 cytokine family, IL-22 is produced by Th17 cells, γδ T cells, NKT cells, as well as innate lymphoid cells (46). Beside regulating host defenses and playing major roles in autoimmune pathological conditions, IL-22 has been shown to promote tissue regeneration and proliferation

of epithelial and stromal cells in numerous tissues including the thymus (46). Although not required for normal thymus organogenesis or maintenance, as no defects were detected in *Il22*−/− mice, constitutive expression of IL-22 (at a circulating range of 35-95 ng/ml of blood) at steady-state leads to thymic involution (48). Under thymic injury however, IL-22 is critical for the endogenous regeneration of thymic tissue and restoration of T-cell development as it promotes TEC proliferation and survival (48). A recent report even suggested that IL-22 might regulate Foxn1 (47), a forkhead box transcription factor critical for TEC development, maintenance, and regeneration (47). Although promising based on the sole available study conducted so far, further investigations are warranted to confirm and highlight the immuno-regenerative potency of this new cytokine.

### 1.5.6 - IL-21: the most recent thymopoiesis licensee

IL-21 is the latest member of the cytokine family sharing the common γ-chain that comprises IL-2, IL-4, IL-7, IL-9, and IL-15 (49). The γ-chain family is central to the establishment and maintenance of the immune system as humans with a mutation in this receptor develop a disease known as X-linked severe combined immunodeficiency (**XSCID**), which is characterized by the absence of T cells and NK cells and the presence of non-functional B cells (50). The early event triggered by IL-21 engagement is the activation of the Janus Kinase 1 and 3 that phosphorylate tyrosine residues of the intracellular regions of the receptor chains (49). These phosphorylated regions serve as docking sites for the SH2 domains of specific signal transducers and activators of transcription (**STAT**) proteins, including STAT1, STAT3, and to a lesser extent STAT5 (49). Among IL-21 sensitive genes, *Gzma* and *Gzmb* encode for granzymes involved in the activity of cytotoxic T cells and natural killer (49). Other genes such as *Bcl6*, *Bim* and *Prdm1* are major

regulator of B-cell survival and differentiation, while *SOCS1/3* encode for negative feedback regulator of cytokine receptor signaling (49). Previous studies have shown that IL-21 has a critical role in generating Th17 and T-follicular helper (**TFH**) cells (51). Furthermore, IL-21 is able to increase interferon-γ (**IFN-γ**) production and cytotoxic activity of TCR-engaged $CD8^+$ T cells (52). Other studies have shown that IL-21 induces the differentiation of B cells into plasma cells and in increasing IgG production (53).

In addition to its pleotropic effects on immune cells, a novel function has been recently described for IL-21 in T-cell development and thymus regeneration (54-56). More specifically, in an attempt to study the effect of the γ-chain cytokine family on thymocytes differentiation *in vitro*, the group of Claude Perrault found that the expression of the IL-21 receptor (**IL-21R**) was strongly up-regulated on DP3 thymocytes ($CD4^+CD8^+TCR\beta^{hi}CD5^{med}$) undergoing positive selection (56). This up-regulation did not stimulate their differentiation (due to absent activation of extracellular signal-regulated kinases (**ERK**) by IL-21) of DP to the SP stage but was essential for DP3 thymocyte expansion (56). The combination of IL-21 with other differentiation-inducing cytokines such as IL-4, IL-7, or IL-13 generated a 3-fold increase in the number of SP CD8 T cells indicating that IL-21 can synergize with other cytokines for increased production of SP CD8 T cells (**FIGURE 4**). In a subsequent study by the same group, administration of IL-21 to mice suffering from acute thymic atrophy triggered by the synthetic glucocorticoïd dexamethasone (**DEX**) accelerated thymic recovery (54). This therapeutic effect was caused by the upregulation of the IL-21R on DP cells after DEX treatment which led to their proliferation. The study also showed that ETP, DN2, and DN3 thymocytes but not TECs express the IL-21R in steady-state conditions and that treatment of isolated DN cells *in vitro* with rIL-21 induced their

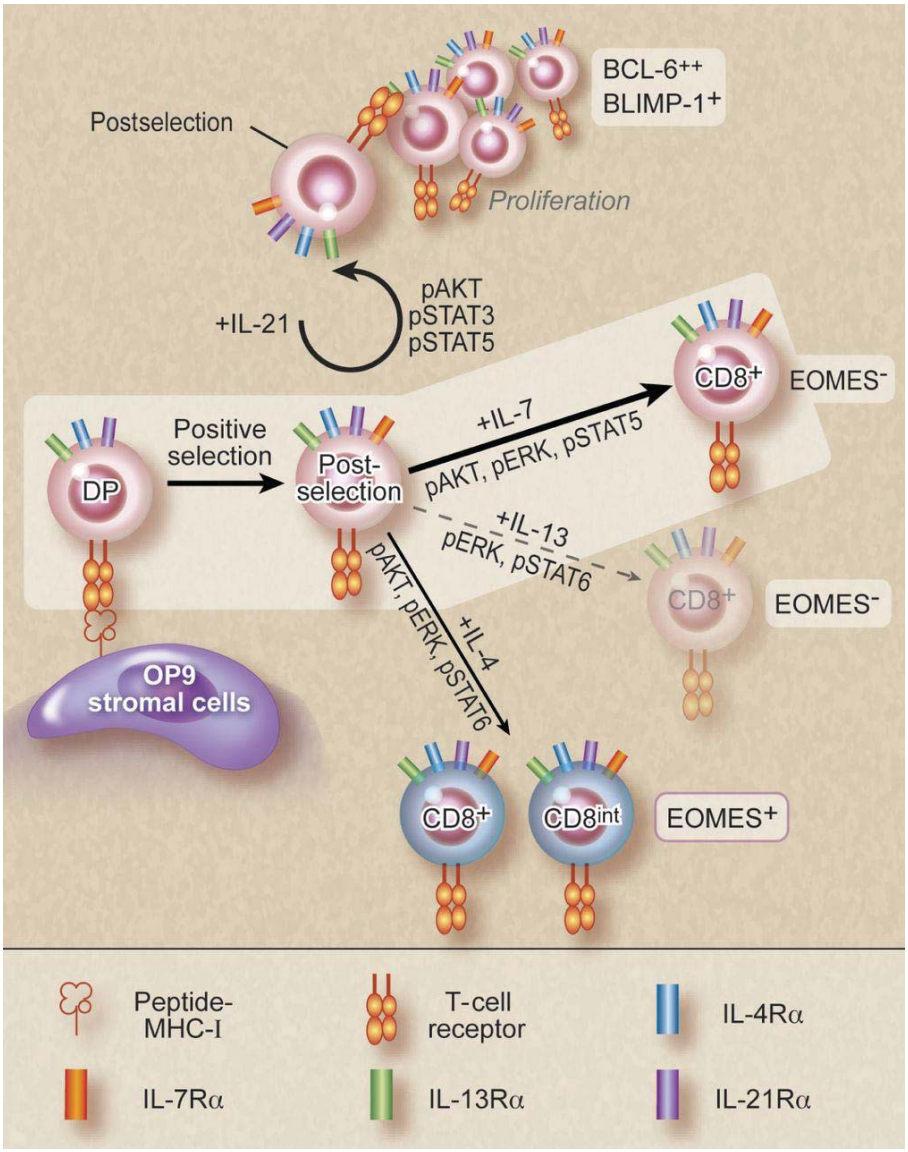



**FIGURE 4**: ***IN VITRO*** **DIFFERENTIATION OF POST-SELECTED THYMOCYTES BY γC-CYTOKINE SIGNALING.**

IL-21 signaling induces expansion of post-selected thymocytes without CD8 lineage commitment, while IL-4, IL-7 and IL-13 promoted CD8 T-cell differentiation. All other tested γc cytokines had no effect on post-selected thymocytes (**Luckey MA *et al.* Blood 2013**) (52).

proliferation (54). The overall significance of these findings epitomizes rIL-21 as a potent pharmacological agent capable of accelerating thymic recovery following iatrogenic-induced thymocyte depletion or natural age-related atrophy.

## 1.6 - Project Hypothesis

Since thymic involution has a negative impact on the health of aged subjects, efforts were put to understand the causes triggering thymic involution in order to prevent or reverse it. Based on recent findings indicating that ETP, DN, and a subset of DP thymocytes express the IL-21R and expand in response to IL-21 treatment both *in vitro* and *in vivo*, we hypothesized that the treatment of aged mice with IL-21 will stimulate thymopoiesis leading to enhanced thymic function and subsequent increase in naïve T-cell output and peripheral TCR diversity. We also speculate that such increase in naïve T cells will render aged mice resistant to cancer due to improved T-cell responses following tumor-associated antigen vaccination.

### 1.6.1 - Objective 1

TO DISSECT THE NATURE OF T-CELL POOLS FOLLOWING IL-21 TREATMENT.

The effect of IL-21 on *de novo* T-lymphopoiesis will be analyzed in three-folds. First, thymic subsets and generation of RTEs will be assessed in PBS- versus IL-21-treated aged mice. Second, flow-cytometry screening of TCRvβ-chains will be conducted to evaluate TCR repertoire diversity. Finally, peripheral T cells will be analyzed to obtain a global estimation of naïve versus memory T-cell pools.

### 1.6.2 - Objective 2

TO EVALUATE THE SENSITIVITY AND SIGNALING ALTERATIONS FOLLOWING TCR STIMULATION OF ENDOGENOUS VERSUS NEWLY DEVELOPED T CELLS.

We presume that injection of IL-21 will increase the frequency of naïve T cells with enhanced TCR sensitivity. To test this hypothesis, T cells derived from treated aged mice will be analyzed for their: i) expression profile of the phosphatases SHP-2, protein tyrosine phosphatase non-receptor type 22 (**PTPN22**), and dual specificity phosphatase (**DUSP5/6**) known to regulate signaling downstream of the TCR, ii) RNA transcripts of mir-181a; the intrinsic modulator of TCR sensitivity, and iii) ERK1/2, lymphocyte-specific protein tyrosine kinase (**Lck**) and zeta-chain-associated protein kinase 70 (**ZAP-70**) phosphorylation. T-cell functionalities in response to TCR stimulation will be assessed as well.

### 1.6.3 - Objective 3

TO EVALUATE THE THERAPEUTIC VALUE OF IL-21 PRE-CONDITIONING THERAPY ON ANTITUMORAL RESPONSES FOLLOWING VACCINATION.

The impact of IL-21 pre-conditioning on cancer vaccination will be tested using the Trp-2 peptide expressed on the surface of B16 melanoma cells. Briefly, aged wild-type C57Bl/6 mice pre-treated with PBS versus IL-21 will undergo peptide-pulsed dendritic cell-based vaccination. The generation of functional effector/memory T cells as well as protective responses to cancer challenge will be analyzed.

In the present project, we propose that biological stimulation of *de novo* intrathymic T-lymphopoiesis using rIL-21 will provide meaningful leads in triggering potent T-cell responses

in the elderly. Furthermore, this unforeseen approach will greatly tailor cancer vaccination to suit aged patients.

**CHAPTRE 2**

***INTERLEUKIN-21 ADMINISTRATION TO AGED MICE REJUVENATES THEIR PERIPHERAL T-CELL POOL BY TRIGGERING DE NOVO THYMOPOIESIS***

## 2.0 - SUMMARY

The vaccination efficacy in the elderly is significantly reduced compared to younger populations due to thymic involution and aged-related intrinsic changes affecting their naïve T-cell compartment. Interleukin (IL)-21 was recently shown to display thymostimulatory properties. Therefore, we hypothesised that its administration to aging hosts may improve T-cell output and thus, restore a competent peripheral T-cell compartment. Indeed, an increase in the production of recent thymic emigrants (RTEs) attributable to intrathymic expansion of early thymic progenitors (ETPs), double-negative (DN)-, and double-positive (DP) thymocytes as well as thymic epithelial cell (TEC) was observed in recombinant (r)IL-21-treated aged mice. In sharp contrast, no alterations in the frequency of bone marrow (BM)-derived progenitors were detected following rIL-21 administration. Enhanced production of naïve T cells improved the T-cell receptor (TCR) repertoire diversity and re-established a pool of T cells exhibiting higher levels of miR-181a and diminished amounts of the TCR-inhibiting phosphatases SHP-2 and DUSP5/6. As a result, stimulation of T cells derived from rIL-21-treated aged mice displayed enhanced activation of Lck, ZAP-70 and ERK, which ultimately boosted their IL-2 production, CD25 expression, and proliferation capabilities in comparison to T cells derived from control aged mice. Consequently, aged rIL-21-treated mice vaccinated using a tyrosinase-related protein 2 (Trp2)-derived peptide exhibited a substantial delay in B16 tumor growth and improved survival. The results of this study highlight the immunorestorative function of rIL-21 paving its use as a strategy for the re-establishment of effective immunity in the elderly.

## 2.1 - INTRODUCTION

In addition for being the key site of T-lymphopoiesis in jawed vertebrates, the thymus maintains a competent peripheral T-cell pool with a broad spectrum of TCR specificities (Lynch *et al.* 2009). It is however well established that immunity declines with aging owing to two key factors impeding thymic function: a defect in the survival and proliferation ability of the pre-thymic hematopoietic progenitor pool coupled to the precocious loss of TECs (Boehm & Swann 2013). These age-related changes, collectively known as thymic involution, represent major driving forces for homeostatic expansion of pre-existing peripheral T cells (Lynch *et al.* 2009). The net outcome culminates in TCR repertoire skewing with a noticeable increase in the number of effector/memory T cells (Zanni *et al.* 2003). Notably, a growing body of literature established that while the size of the peripheral T-cell compartment remains unchanged throughout aging, an increase in post-thymic life span of T cells takes place consequently leading to the emergence of T-cell intrinsic defects (Haynes & Swain 2006; Maue *et al.* 2009; Tsukamoto *et al.* 2009). For instance, naïve $CD4^+$ T cells derived from aged mice display defects in TCR threshold calibration, do not readily form immunological synapses and have a marked reduction in the recruitment of TCR-associated signaling molecules when compared to younger mice (Garcia & Miller 1997; Tamir *et al.* 2000; Garcia & Miller 2001; Garcia & Miller 2002). Furthermore, increased expression of inhibitory receptors such as PD1, LAG3, 2B4 and CD160 were observed on the surface of aging $CD8^+$ T cells (Decman *et al.* 2012) while both IL-2 secretion and proliferation potential are limited in naïve $CD4^+$ and $CD8^+$ T cells derived from aged mice (Eaton *et al.* 2004). Thus, stifled thymopoiesis combined to global qualitative changes affecting the aging peripheral T-cell pool limits the host's ability to mount effective responses against new antigenic challenges and accounts for the eroded immunity commonly observed in the elderly.

Primarily produced by activated $CD4^+$ T cells, IL-21 is a prominent member of the common γ-chain family of cytokines (Spolski & Leonard 2014). In addition to its wide ranging effects on immune cells, IL-21 overexpression *in vivo* triggers potent expansion of BM-derived progenitors (Ozaki et al. 2006). Furthermore, we recently reported a novel mitogenic function for IL-21 on peptide-mediated TCR-engaged DP thymocytes using a newly developed *in vitro* co-culture system designed for T-cell differentiation (Rafei *et al.* 2013b). Likewise, rIL-21 administration to mice with glucocorticoïd-induced thymic atrophy dramatically accelerates thymic function recovery by stimulating the proliferation of ETPs, DN and positively selected DP thymocytes (Rafei *et al.* 2013a). Such unprecedented thymopoiesis-supporting function suggests that rIL-21 is indeed a promising therapeutic tool endowed with the capacity of improving T-cell output in aged hosts owing to the expression of the IL-21 receptor (IL-21R) on both BM and thymic progenitors (Ozaki *et al.* 2006; Rafei *et al.* 2013a; Rafei *et al.* 2013b). We wished therefore to scrutinize whether rIL-21 administration to aged mice can rejuvenate their T-cell immunity by targeting *de novo* thymopoiesis as a mean to enhance their anti-tumoral response following vaccination.

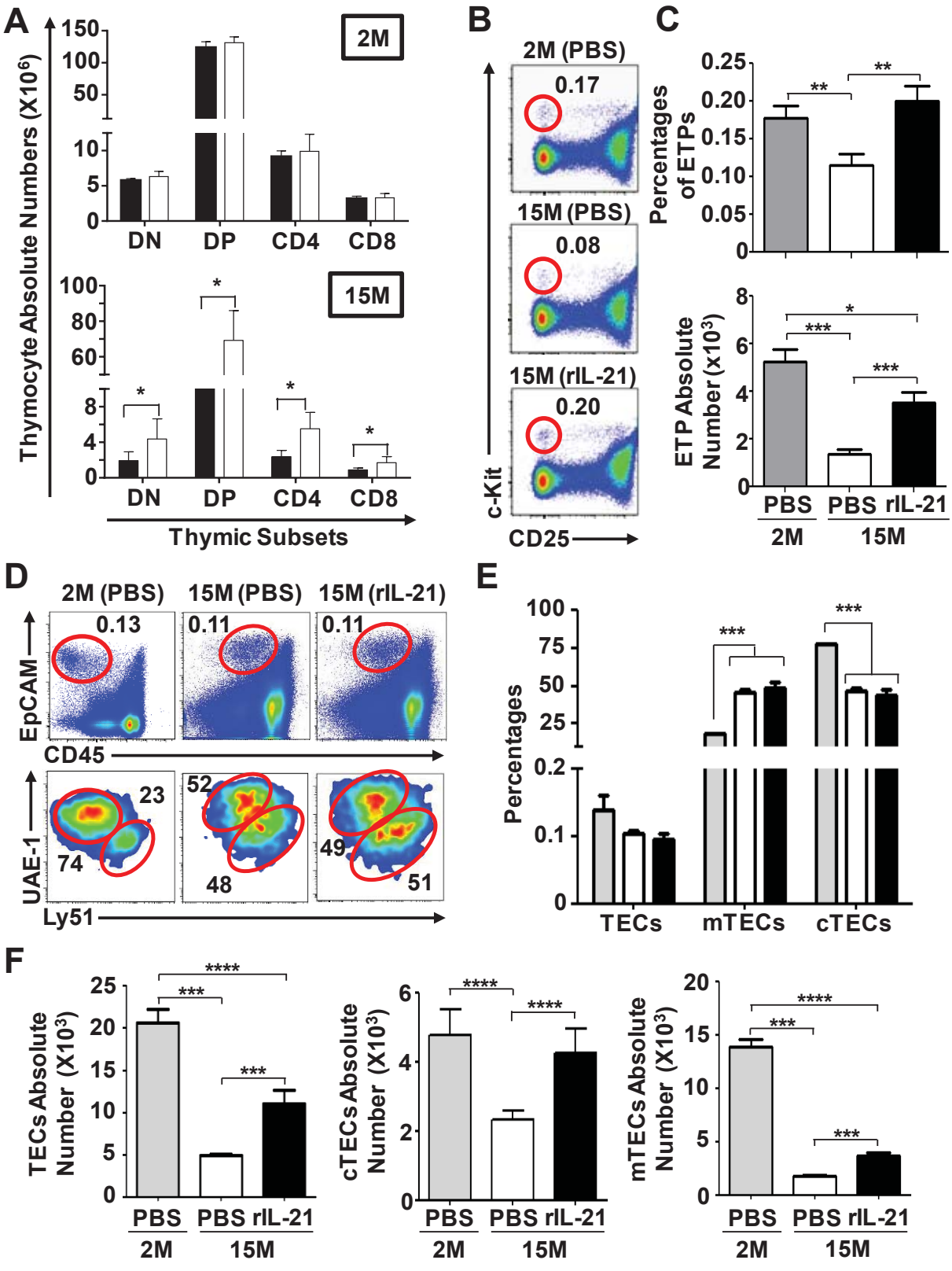
## 2.2 - RESULTS

**Administration of rIL-21 enhances thymopoiesis in aged mice.**

To ensure maximal thymopoiesis-stimulating effects *in vivo*, we first conducted a dose response study in young (2 months - 2M) versus old (15 months - 15M) RAG2p-GFP mice by intraperitoneally (IP) administering rIL-21 (Fig. S5A). The use of the RAG2p-GFP model allows to easily assess *de novo* thymopoiesis as expression of the GFP transgene, marking newly developed T cells, is controlled by the *Rag2* promoter activity (Monroe *et al.* 1999; Yu *et al.* 1999; Rafei *et al.* 2011). Thymic analysis one week following the last injection revealed a progressive increase in total thymocyte absolute count in 15M but not 2M old rIL-21-treated mice with an optimal response rate achieved at a dose of 50ug/Kg (Fig. S5B). Similarly, only aged mice receiving rIL-21 exhibited an increase in the counts of all thymic subsets (DN, DP and single-positive) (Fig. 5A) including ETPs (Fig. 5B-C). Even though the percentage of $GFP^+$ thymocytes remained unchanged in all studied groups (Fig. S5C), the increased thymic count observed in the rIL-21-treated aged mice was sustained over a three weeks period post-cytokine administration (Fig S5D). To determine whether rIL-21-enhanced thymopoiesis involves the expansion of BM progenitors, which could have increased their migration rate to the thymus, we next monitored the frequency of $LSK^+$ cells ($Lin^-Sca1^+c\text{-}Kit^+$) and its sub-populations including the long-term (LT; $Lin^-Sca1^+c\text{-}Kit^+CD34^-CD135^-$) and short-term (ST; $Lin^-Sca1^+c\text{-}Kit^+CD34^+CD135^-$)-hematopoietic stem cells (HSCs), as well as multi-potent progenitors (MPPs; $Lin^-Sca1^+c\text{-}Kit^+CD34^+CD135^+$) following rIL-21 treatment. Despite IL-21R expression on the surface of wild-type (WT) LT-, ST-HSCs and MPPs (Fig. S6A), the overall proportion of $LSK^+$ cells (Fig. S6B) or its sub-populations (Fig. S6C) was not affected by rIL-21 administration.

**FIGURE 5. ADMINISTRATION OF rIL-21 PROMOTES *DE NOVO* THYMOPOIESIS IN AGED BUT NOT YOUNG MICE.**

A) Counts of thymocyte sub-populations at 50ug/kg of rIL-21. B) Representative flow-cytometry analysis of ETPs. C) Absolute count of ETPs derived from 2M (PBS□), 15M (PBS□) or 15 (rIL-21■). D-E) Percentages of total, cTECs and mTECs in 2M (PBS□), 15M (PBS□) or 15M (rIL-21■). F) All rIL-21-treated aged mice display enhanced counts of total, cTECs and mTECs in comparison to PBS-treated aged mice. All data are representative of three independent experiments (n = 5/group with *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001).


A
2M
15M
Thymocyte Absolute Numbers (X10⁶)
DN
DP
CD4
CD8
Thymic Subsets
B
2M (PBS)
0.17
15M (PBS)
0.08
15M (rIL-21)
0.20
c-Kit
CD25
C
Percentages of ETPs
ETP Absolute Number (x10³)
PBS
PBS rIL-21
2M
15M
D
2M (PBS)
15M (PBS)
15M (rIL-21)
0.13
0.11
0.11
EpCAM
CD45
UAE-1
Ly51
23
74
52
48
49
51
E
Percentages
TECs
mTECs
cTECs
F
TECs Absolute Number (X10³)
cTECs Absolute Number (X10³)
mTECs Absolute Number (X10³)

Likewise, no increase in the number of $LSK^+$ sub-populations (Fig. S6D) nor in the more differentiated CLP ($Lin^-IL\text{-}7R^+Sca1^+c\text{-}Kit^+$) population (Fig. S6E) was observed.

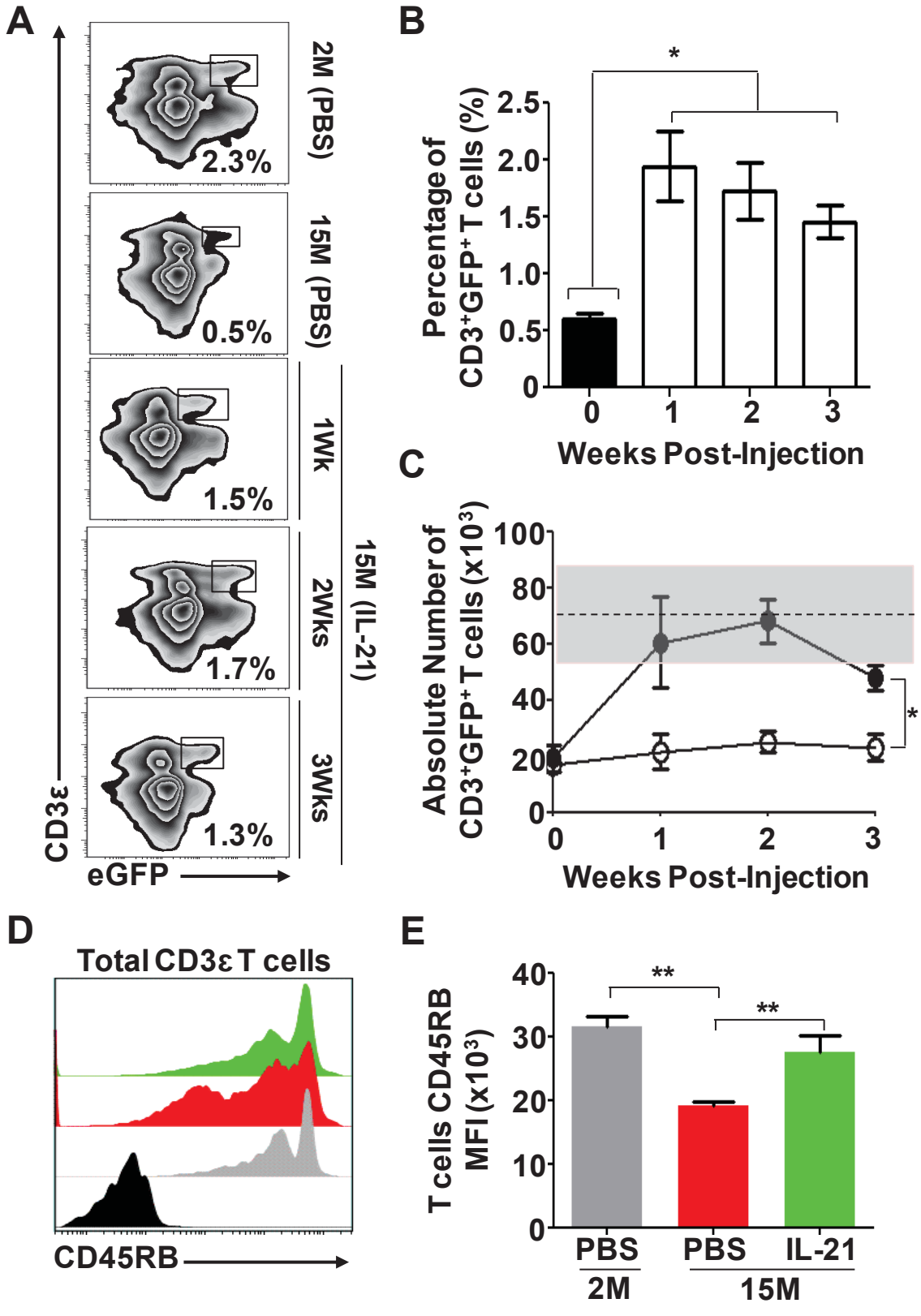
We recently reported that TECs are devoid of IL-21R (Rafei *et al.* 2013a). Therefore, we presumed that the thymic effects observed following rIL-21 infusion strictly affect hematopoietic cells. Indeed, when given to aged mice, rIL-21 does not fluctuate the frequency of total TECs ($EpCAM^+CD45^-$), nor it did affect the ratio of cortical (c)TEC ($EpCAM^+CD45^-UAE\text{-}1^-Ly51^+$) relative to medullary (m)TECs ($EpCAM^+CD45^-UAE\text{-}1^+Ly51^{-/lo}$) populations (Fig. 5D-E). Conversely, absolute counts analysis showed marked improvements in the stromal compartment as total, cTEC and mTEC populations were significantly higher in rIL-21-treated aged mice compared to those derived from the PBS group (Fig. 5F). Higher production of IL-7 /thymus was also noticed in the rIL-21-treated aged mice compared to the control group (Fig. S5E). These data suggest that rIL-21 administration is beneficial to aged mice by directly targeting thymopoiesis *in situ* without triggering the expansion of BM-derived $LSK^+$ cells.

**Physiological levels of RTE are restored in aged mice following rIL-21 treatment.**

To interrogate the functional relevance of rIL-21-enhanced thymopoiesis on the peripheral T-cell pool of aged RAG2p-GFP mice, we next assessed the percentage of circulating RTEs mirrored by the level of peripheral $GFP^+CD3\varepsilon^+$ T cells. In contrast to 2M old animals, where RTEs represent roughly 2.3% of total circulating lymphocytes, lower percentages (~0.5%) are detected in the peripheral blood of 15M PBS-treated aged mice (Fig. 6A). Following rIL-21 treatment, the percentage of $GFP^+CD3\varepsilon^+$ T cells reached a range of 1.3-1.7% over a period of three weeks post-

**FIGURE 6. INCREASED LEVELS OF CIRCULATING RTES IN rIL-21-TREATED AGED MICE.**

A) Representative flow-cytometry analysis of RTEs ($CD3^+GFP^+$ T cells) in peripheral blood of young (2M) or aged (15M) mice 1, 2, or 3 weeks post-rIL-21 administration. Young mice (2M) treated with PBS were used as comparative positive controls. B-C) Analysis of overall percentages (B) and counts (C) of RTEs in the weeks following rIL-21 administration. The gray zone represents the RTE level calculated using 2M young mice (n=10) and displayed as the average RTE number $\pm$ two S.D. Filled circles represent rIL-21-treated aged mice. D) Representative flow-cytometry analysis of CD45RB on the surface of all $CD3^+$ T cells derived from 2M (PBS□), 15M (PBS■) and 15M (rIL-21■). CD45RB isotype is displayed in black. E) Compiled MFIs for CD45RB expression in treated mice. All data are representative of three independent experiments (n = 5/group with *p<0.05 and **p<0.01).


A
2M (PBS)
15M (PBS)
1Wk
2Wks
3Wks
15M (IL-21)
2.3%
0.5%
1.5%
1.7%
1.3%
CD3ε
eGFP
B
Percentage of CD3+GFP+ T cells (%)
2.5
2.0
1.5
1.0
0.5
0
*
0
1
2
3
Weeks Post-Injection
C
Absolute Number of CD3+GFP+ T cells (x10^3)
100
80
60
40
20
0
*
0
1
2
3
Weeks Post-Injection
D
Total CD3ε T cells
CD45RB
E
T cells CD45RB MFI (x10^3)
40
30
20
10
0
**
**
PBS
2M
PBS
IL-21
15M

cytokine treatment (Fig. 6A-B) with absolute numbers attaining physiological levels according to RTE counts calculated using blood derived from young mice (Fig. 6C).

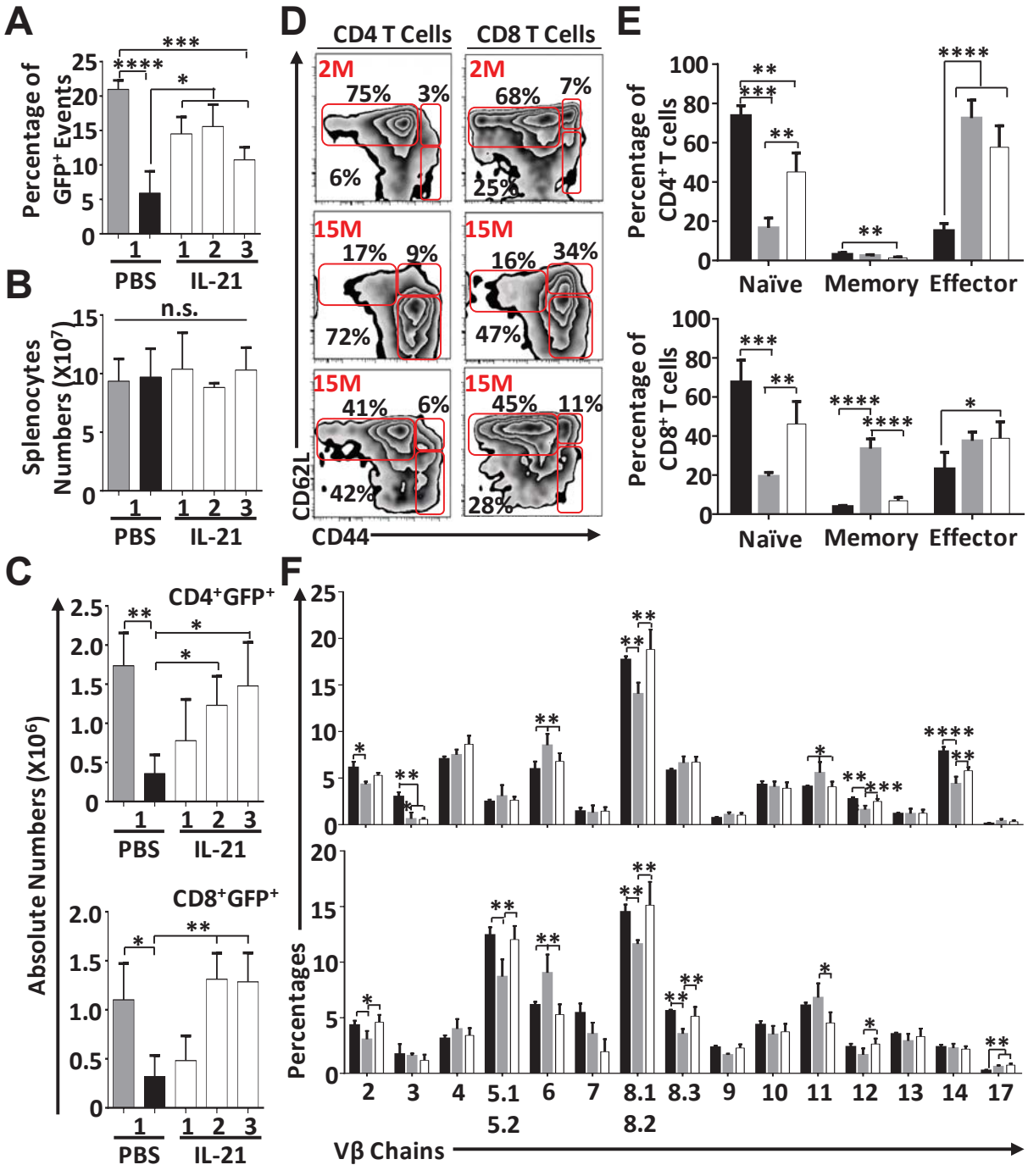
With increased encountered antigens and declined RTE levels, qualitative changes in the phenotype of peripheral T-cell composition occur with aging (Boursalian *et al.* 2004). More specifically, the expression of various cell surface makers including the glycoprotein CD45RB is down-regulated as T cells become activated and progress from a naïve to a memory phenotype (Tough & Sprent 1994). We therefore hypothesized that enhancing RTE generation in aged mice would increase the overall expression pattern of CD45RB on peripheral T-cell pool and found that it was indeed the case in aged mice treated with rIL-21 as depicted by histogram overlaps (Fig. 6D) and compiled mean fluorescent intensities (MFIs) (Fig. 6E). We therefore conclude that rIL-21 treatment enhances the *de novo* generation of RTEs, which incorporate the peripheral T-cell pool of aged mice.

**The nature of aging T-cell pool is greatly affected by rIL-21 administration.**

Following thymic egress, RTEs continue their maturation in the periphery to eventually become fully competent mature naïve T cells (Boursalian *et al.* 2004). To do so, they require access to secondary lymphoid organs (SLO) as a mean to encounter other cell types and cytokines required for their maturation (Houston *et al.* 2008). Although the percentage of $GFP^+$ T cells increased significantly in the spleen of rIL-21-treated aged mice in the three weeks following cytokine treatment (Fig. 7A), the overall number of splenocytes remained steady (Fig 7B). Further in depth analysis revealed a progressive time-dependent increase in the absolute counts of $GFP^+CD4^+$ and $GFP^+CD8^+$ T cells in the spleens of rIL-21-treated aged mice (Fig. 7C)

**FIGURE 7. AGED MICE TREATED WITH rIL-21 DISPLAY INCREASED PROPORTION OF NAÏVE T CELLS WITH ENHANCED TCR DIVERSITY.**

A) Percentages of $GFP^+$ events in the spleen of 2M (PBS ▨), 15M (PBS □), and 15M (rIL-21 ■) aged mice. B) Splenocytes counts in all experimental groups. C) Absolute counts of $CD4^+GFP^+$ and $CD8^+GFP^+$ T cells in 2M (PBS ▨), 15M (PBS □) and 15M (rIL-21 ■) treated mice. D) A representative flow-cytometry analysis of naïve ($CD62L^{hi}$, $CD44^{lo}$), memory ($CD62L^{hi}CD44^{hi}$) and effector ($CD62L^{lo}CD44^{hi}$) T cells in all experimental groups. E) Compiled percentages of all three sub-populations in $CD4^+$ (top panel) and $CD8^+$ (lower panel) T cells. F) Flow-cytometry analysis of 15 TCRVβ-chains using peripheral $CD4^+$ (top panel) or $CD8^+$ (lower panel) T cells. All data are representative of three independent experiments (n = 5/group with *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$).


A
Percentage of GFP⁺ Events
PBS
IL-21
B
Splenocytes Numbers (X10⁷)
n.s.
C
CD4⁺GFP⁺
CD8⁺GFP⁺
Absolute Numbers (X10⁶)
D
CD4 T Cells
CD8 T Cells
2M
15M
CD62L
CD44
E
Percentage of CD4⁺T cells
Percentage of CD8⁺T cells
Naïve
Memory
Effector
F
Percentages
Vβ Chains

suggesting that SLO-resident aged T cells were displaced by newly migrating RTEs. We next examined by flow-cytometry the differentiation stages of spleen-derived $CD4^+$ and $CD8^+$ T cells and found increased abundance of T cells with a naïve phenotype ($CD44^{lo}CD62L^{hi}$) in rIL-21-treated aged mice bolstering the notion of rIL-21-mediated enhanced T-cell output (Fig. 7D-E).

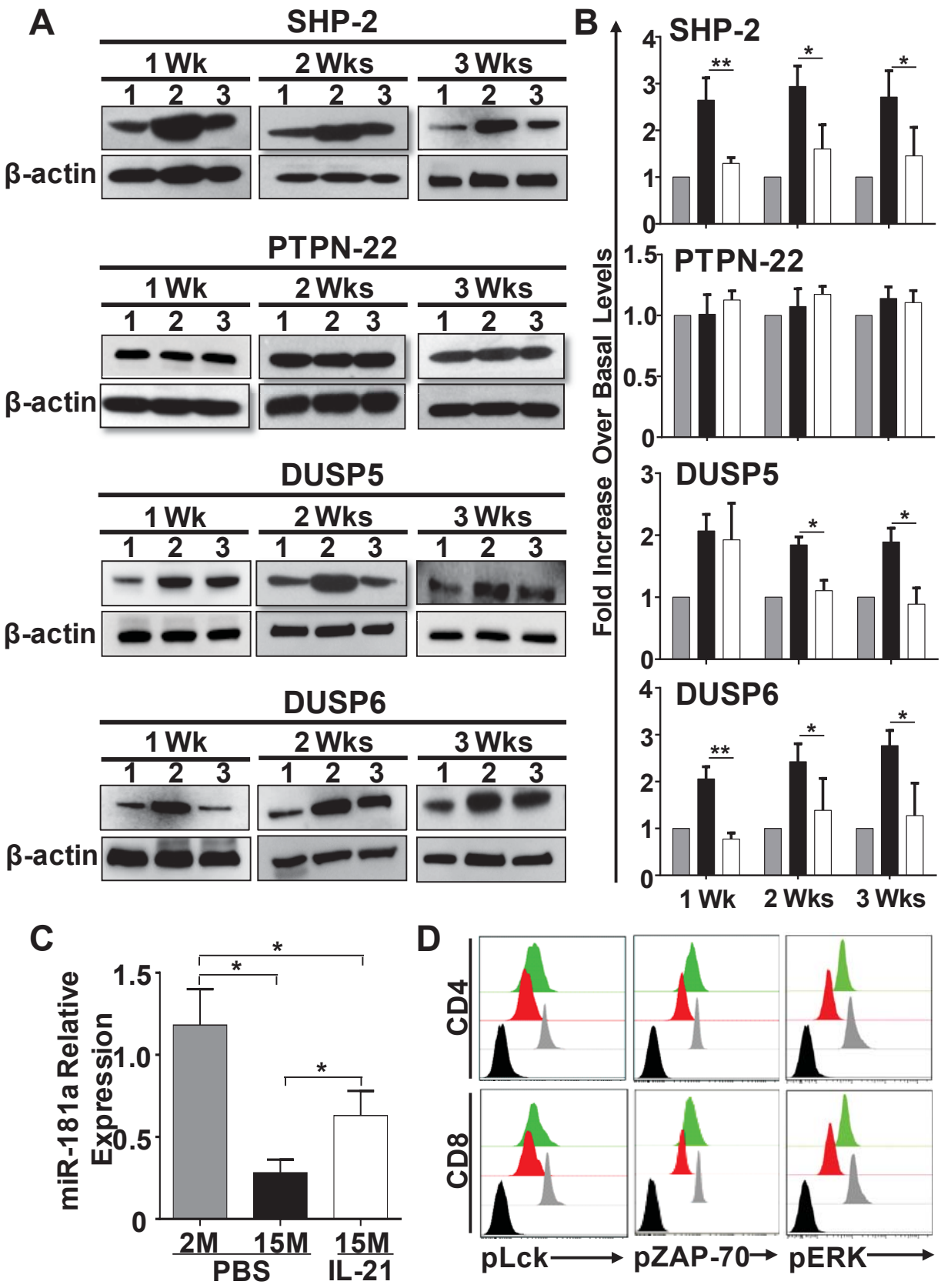
Given that thymic involution compromises the TCR repertoire (Yager *et al.* 2008; Ahmed *et al.* 2009), we continued our analysis by investigating the effect of rIL-21 administration on TCR diversity. To address this question, the expression profile of 15 TCRVβ-chains was analyzed on the surface of spleen-derived $CD4^+$ and $CD8^+$ T cells collected from treated mice. Significant improvements were observed in the proportion of Vβ2-, 6-, 8.1/8.2-, 11-, 12- and 14-expressing $CD4^+$ T cells as well as Vβ2-, 5.1/5.2-, 6-, 8.1/8.2-, 8.3-, 11- and 12-expressing $CD8^+$ T cells following rIL-21 administration to aging mice (Fig. 7F). These results indicate that rIL-21-mediated *de novo* RTE generation is associated with major qualitative changes in the peripheral T-cell pool of aged mice including improved TCR diversity.

**Characterizing the biochemical responses of T cells.**

Thymic involution cannot solely account for impaired immune responses as additional T-cell intrinsic defects appear in aging naïve T cells due to prolonged post-thymic life span (Haynes & Swain 2006; Maue *et al.* 2009; Tsukamoto *et al.* 2009). More specifically, TCR activation is blunted with aging due to increased cytoplasmic concentration of phosphatases known for inhibiting TCR signaling (Li *et al.* 2012). Given such fluctuation in TCR threshold calibration, we next explored whether the previously observed changes in the nature of the peripheral T-cell pool induced by rIL-21 affect the expression levels of the TCR-targeting phosphatases SHP-2,

**FIGURE 8. THE PERIPHERAL POOL OF T CELLS IN rIL-21-TREATED AGED MICE DISPLAY IMPROVED TCR SIGNALING RESPONSES.**

A) Representative western-blot analyses of phosphatases at 1, 2 or 3 weeks post-treatments of 2M PBS (1), 15M PBS (2) and 15M rIL-21 (3) aged mice. β-actin was used as internal loading control. B) Compiled densitometry analysis of SHP-2, PTPN-22 and DUSP5/6 phosphatase expression levels. The displayed groups are: 2M (PBS□); 15M (PBS■); and 15M (rIL-21□) aged mice. C) qPCR analysis of miR-181a in freshly isolated T cells from 2M (PBS□); 15M (PBS■); and 15M (rIL-21□) treated mice. D) Representative intracellular flow-cytometry staining of pLck, pZAP-70 and pERK in 2M (PBS□); 15M (PBS■ ); and 15M (rIL-21■) aged mice. Non-stimulated T cells derived from young 2M mice are displayed by black histograms. All data are representative of three independent experiments (n = 5/group with *$p<0.05$, and **$p<0.01$).


A
SHP-2
1 Wk
2 Wks
3 Wks
1 2 3 1 2 3 1 2 3
β-actin
PTPN-22
DUSP5
DUSP6
B
SHP-2
PTPN-22
DUSP5
DUSP6
Fold Increase Over Basal Levels
1 Wk
2 Wks
3 Wks
C
miR-181a Relative Expression
2M
15M PBS
15M IL-21
D
CD4
CD8
pLck
pZAP-70
pERK

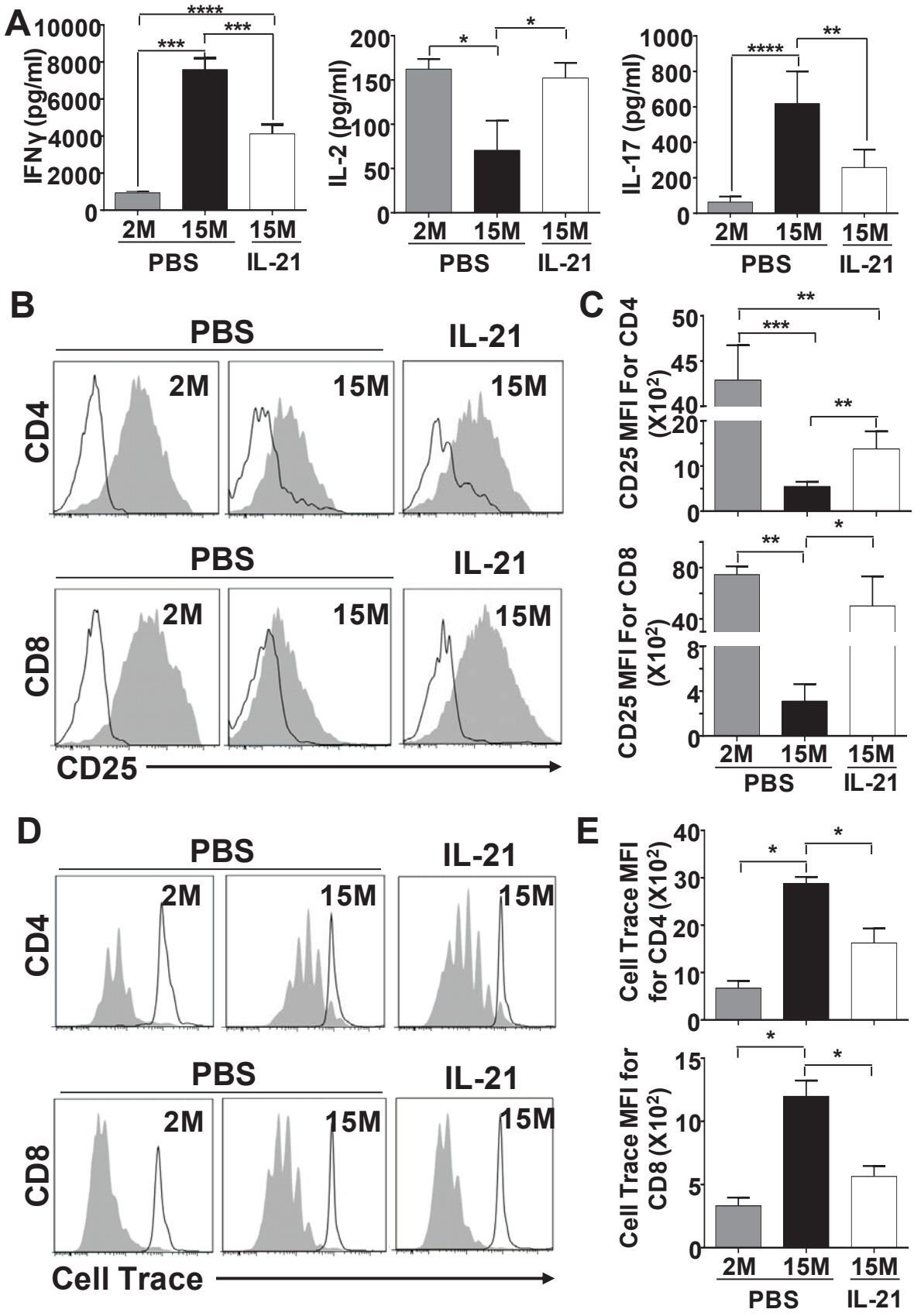
PTPN-22 and DUSP5/6. Delineation of phosphatase levels by western-blotting (Fig. 8A) and densitometry-based quantification (Fig. 8B) revealed diminished SHP-2 and DUSP5/6 levels in T cells derived from rIL-21-treated aged mice at all tested time points. Only PTPN-22 remained unchanged in all groups throughout treatments (Fig. 8A-B). Previous studies conducted in mice and humans demonstrated that SHP-2 and DUSP5/6 were among several phosphatases controlled by miR-181a; a ~22nt microRNA molecule capable of repressing the translation of over 40 phosphatases (Li *et al.* 2007). Consistently, the progressive decline in miR-181a levels observed in aging human naïve $CD4^+$ T cells dovetails the decreased T-cell responsiveness following TCR stimulation (Li *et al.* 2012). To examine the possibility that rIL-21 could have reversed such defect through enhanced generation of RTEs expressing normal levels of miR-181a, quantitative (q)PCR studies were conducted using T cells sorted from spleens of treated mice. Our analysis revealed a 2-3 fold increase in miR-181a levels in T cells derived from rIL-21-treated aged mice (Fig. 8C), which is consistent with the diminished SHP-2 and DUSP5/6 levels observed earlier (Fig 8A-B). We next tested the responses of T cells derived from treated mice by probing early signaling events triggered by TCR stimulation. Although not efficient as younger lymphocytes, $CD4^+$ or $CD8^+$ T cells derived from rIL-21-treated aged mice displayed higher Lck, ZAP-70 and ERK phosphorylation compared to control PBS aged mice as shown by phospho-flow analysis (Fig. 8D). Taken collectively, these data indicate that enhanced TCR responses in the pool of T cells derived from rIL-21-treated aged mice is attributable to the increase in the proportion of naïve T cells displaying lower SHP-2 and DUSP5/6 phosphatase levels owing to improved miR-181a expression.

**rIL-21 administration improves the biological responses of T cells derived from aged mice.**

The dramatic reduction in naïve T-cell output coupled to the relative increase in the proportion of effector and central memory T cells in aged mice negatively impact T-cell responses to neo-antigens (Yager *et al.* 2008). Repeated observations revealed that beside scarcity of T-cell precursors in the pre-immune repertoire, IL-2 production, cell surface expression of CD25 and T-cell proliferation are all greatly reduced in aging hosts (Haynes & Swain 2006; Maue *et al.* 2009; Tsukamoto *et al.* 2009). Elevated secretion levels of pro-inflammatory cytokines such as interferon (IFN)γ caused by the accumulation of $CD44^{hi}$ T cells were also reported to play a role in accelerating age-related immunosenescence (Zhang *et al.* 2002; Decman *et al.* 2012). The observations made so far led us to speculate that the beneficial effect of rIL-21 on thymopoiesis in aged mice should improve the effector function of their rejuvenated T-cell pool. To test this hypothesis, spleen-derived $CD3\varepsilon^{+}$ T cells were isolated three weeks following the last cytokine treatment (to ensure that they had completed their necessary post-thymic maturation) and stimulated with CD3-CD28 beads (Berkley *et al.* 2013). Not only were IFNγ and IL-2 secretion profiles by TCR-stimulated T cells derived from rIL-21-treated mice comparable to younger animals, but a striking decrease in IL-17 production was observed as well (Fig. 9A). In addition, the intensity of CD25 cell surface expression on spleen-purified TCR-stimulated $CD4^{+}$ or $CD8^{+}$ T cells and their proliferation rates were comparable between young and rIL-21-treated aged mice as determined by flow-cytometry (Fig 9B, D) and compiled MFIs data (Fig. 9C, E). To gain further insights, we quantified by qPCR the expression level of several genes known to influence the differentiation and/or effector function of T cells. Although no difference were observed in *Nfat*, *Ap-1* and *Gata3* expression in all tested groups, a decrease in *T-bet* and *RORC* (encoding for RORγt) transcript levels was detected in T cells derived from rIL-21-treated aged mice

**FIGURE 9. ENHANCED BIOLOGICAL RESPONSES OF PERIPHERAL T-CELL POOL DERIVED FROM rIL-21-TREATED AGED MICE.**

A) IFNγ, IL-2 and IL-17 secretion quantification from T cells isolated from treated groups and stimulated with CD3/CD28 dynabeads for 48hrs. B) Representative flow-cytometry analysis of CD25 cell surface expression on $CD4^+$ (top panel) and $CD8^+$ (lower panel) T cells. C) Compiled MFIs for CD25 expression on $CD4^+$ and $CD8^+$ T cells. D) Representative flow-cytometry analysis of cell trace dilution following TCR stimulation of $CD4^+$ (top panel) and $CD8^+$ (lower panel) T cells derived from treated animals with CD3/CD28 beads. E) Compiled MFIs for cell trace dilution of $CD4^+$ and $CD8^+$ T cells following stimulation. All data are representative of three independent experiments (n=5/group with *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001).


A
IFNγ (pg/ml)
IL-2 (pg/ml)
IL-17 (pg/ml)
2M
15M
15M
PBS
IL-21
B
PBS
IL-21
CD4
CD8
CD25
C
CD25 MFI For CD4 (X10²)
CD25 MFI For CD8 (X10²)
D
CD4
CD8
Cell Trace
E
Cell Trace MFI for CD4 (X10²)
Cell Trace MFI for CD8 (X10²)

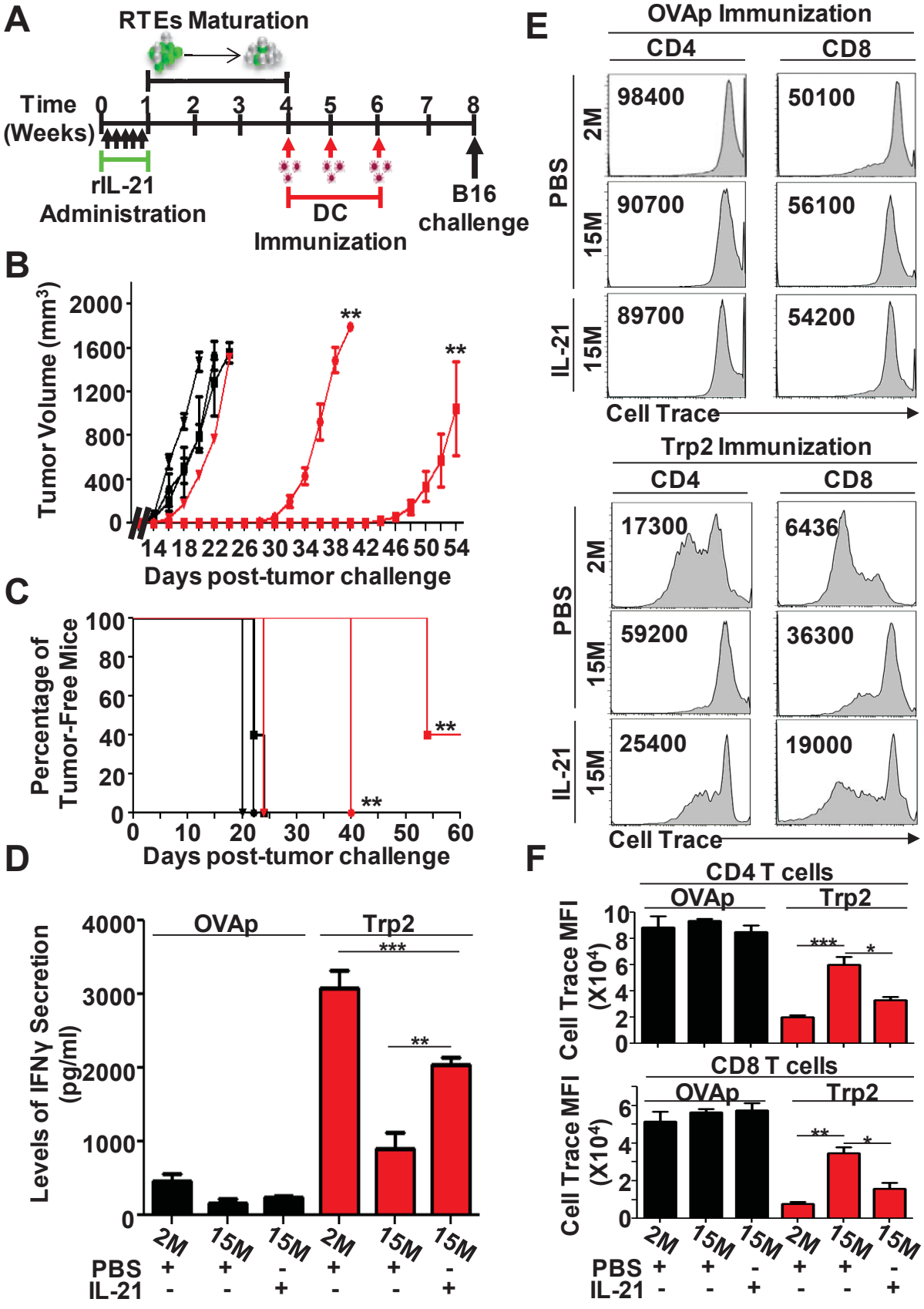
compared to aged PBS control mice (Fig. S7). *Foxp3* expression level, on the other hand, was equivalent between PBS- and rIL-21-treated aged mice but significantly higher than in younger mice (Fig.S7). These qPCR results are consistent with the observed decrease in IFNγ (*T-bet*) and IL-17 (*RORC*) secretion levels and are particularly interesting as several investigations have associated IL-21 to autoimmune diseases (Korn *et al.* 2007; Peluso *et al.* 2007; Attridge *et al.* 2012). Further analyses conducted on rIL-21-treated aged mice showed no unusual signs of inflammation or autoimmunity (Fig. S8). More specifically, the spleen size and weight of PBS- or rIL-21-treated aged mice were similar to younger mice (Fig. S8A). Total serum IgG levels were however lower in young mice but comparable in both PBS- and rIL-21-treated animals indicating an age-related factor at play independent of rIL-21 treatment (Fig. S8B). Furthermore, no immune infiltrates were detected in lungs, liver or kidneys of aged mice receiving rIL-21 (Fig. S8C). In agreement with previous studies reporting accumulation of $CD25^{+}CD4^{+}$ $T_{regs}$ in the periphery and SLO of aging mice and humans (Lages *et al.* 2008), we detected a significantly higher percentage and absolute number of spleen-derived $T_{regs}$ in both PBS- and rIL-21-treated aged mice when compared to their younger counterparts (Fig. S8D-E). Globally, these findings indicate that rIL-21-mediated boosting of T-cell output in aged mice corrects the T-cell dysfunctionalities commonly seen with aging without inducing signs of autoimmunity.

**Enhanced vaccine-elicited anti-tumoral response in rIL-21-treated aged mice.**

To evaluate the effectiveness of our T-cell rejuvenation therapy, a proof-of-concept vaccination study was conducted using an experimental melanoma antigen (Parkhurst *et al.* 1998). Briefly, aged mice were first treated with PBS or rIL-21 according to our established protocol then left

**FIGURE 10. T-CELL REJUVENATION OF AGED MICE ENHANCES THEIR ANTI-TUMORAL IMMUNITY.**

A) Schematic diagram representing the timeline used for vaccination. B-C) Tumor volumes and percentage of tumor-free mice post-B16 tumor cell challenge in SIINFEKL-DC-vaccinated 2M (PBS■); 15M (PBS▼); 15M (rIL-21●) or Trp2-DC-vaccinated 2M (PBS■); 15M (PBS▼); 15M (rIL-21●). D) IFNγ quantification by ELISA following *in vitro* Trp2-stimulation of T cells derived from vaccinated animals. E) Representative flow-cytometry analysis of cell trace dilution in $CD4^+$ and $CD8^+$ T cells derived from vaccinated animals following *in vitro* Trp2 stimulation. F) Compiled MFIs for cell trace dilutions in $CD4^+$ (top panel) and $CD8^+$ (lower panel) T cells following *in vitro* Trp2 stimulation. All data are representative of three independent experiments (n = 5/group with *p<0.05, **p<0.01, and ***p<0.001).


A
RTEs Maturation
Time (Weeks)
0 1 2 3 4 5 6 7 8
rIL-21 Administration
DC Immunization
B16 challenge
B
Tumor Volume (mm3)
2000
1600
1200
800
400
0
14 18 22 26 30 34 38 42 46 50 54
Days post-tumor challenge
**
**
C
Percentage of Tumor-Free Mice
100
80
60
40
20
0
0 10 20 30 40 50 60
Days post-tumor challenge
**
**
D
Levels of IFNγ Secretion (pg/ml)
4000
3000
2000
1000
0
OVAp
Trp2
***
**
2M 15M 15M 2M 15M 15M
PBS + + - + + -
IL-21 - - + - - +
E
OVAp Immunization
CD4
CD8
PBS
IL-21
2M
15M
98400
50100
90700
56100
89700
54200
Cell Trace
Trp2 Immunization
CD4
CD8
17300
6436
59200
36300
25400
19000
Cell Trace
F
CD4 T cells
OVAp
Trp2
Cell Trace MFI (X10⁴)
10
8
6
4
2
0
***
*
CD8 T cells
OVAp
Trp2
Cell Trace MFI (X10⁴)
6
4
2
0
**
*
2M 15M 15M 2M 15M 15M
PBS + + - + + -
IL-21 - - + - - +

for three weeks to allow for RTE maturation (Fig. 10A). All mice were then vaccinated weekly (for a total of three injections) using *in vitro* generated BM-derived mature DCs (Fig. S9) pulsed with SIINFEKL (a control epitope derived from chicken ovalbumin) or SVYDFFVWL peptides (the experimental epitope derived from the melanoma differentiation antigen Trp2). Two weeks following the last DC boost, mice where challenged subcutaneously (SC) with syngeneic B16 tumor cells and monitored thereafter for tumor growth and survival. Although all SIINFEKL-vaccinated mice died within the same time frame (day 20-24) with no statistically significant difference between young, PBS- or rIL-21-treated mice, a slower tumor growth (Fig. 10B) and a significant delay in survival (Fig. 10C) were observed in the Trp2-vaccinated rIL-21-treated aged mice in comparison to the Trp2-vaccinated PBS control group (survived up to 40 versus 22 days respectively). We then retrieved the spleens of mice from each group and analyzed their IFNγ production level and proliferation following Trp2 re-stimulation *in vitro*. All Trp2-vaccinated animals responded to peptide re-stimulation with a significant increase in IFNγ response achieved in rIL-21-treated aged mice compared to control PBS aged mice (Fig. 10D). Similarly, enhanced proliferation responses were observed in the rIL-21-treated aged mice as evaluated by cell trace dilution overtime by flow-cytometry (Fig. 10E) and compiled MFIs data (Fig. 10F). In sum, these data clearly show that rIL-21 pre-conditioning of aged mice leads to marked improvements in the control of B16 tumor growth following Trp2 vaccination.

## 2.3 - DISCUSSION

Thymic involution deprives aged hosts from a competent immune system capable of effectively responding to vaccination and invading pathogens (Boehm & Swann 2013). Although some aspects of age-related decline in T-cell responses reflect systemic changes, others are due to cell-intrinsic defects (Haynes & Swain 2006; Maue *et al.* 2009; Tsukamoto *et al.* 2009). We show in this report that administration of rIL-21 enhances thymopoiesis in aged mice through expansion of both the stromal and responsive thymocytes compartments without the induction of any apparent pathology in peripheral organs. Consequently, a competent peripheral lymphoid pool containing larger proportion of naïve $CD4^+$ and $CD8^+$ T cells ($CD62L^{hi}CD44^{lo}$) displaying potent effector functions in response to TCR stimulation was restored. This increase in the availability and potency of naïve T cells augmented the responsiveness of aged mice to Trp2 vaccination and accounts for their improved survival in response to B16 tumor challenge (Fig. S10).

T-lymphopoiesis remains functional at older age albeit to a limited extent (Hale *et al.* 2006). As the thymus lacks self-renewing progenitors, it heavily relies on sustained seeding with BM-derived CLPs and/or ETPs (Min *et al.* 2004). Unfortunately however, BM-derived progenitor numbers decline markedly with age due to increased apoptosis rates as well as reduced proliferative capacities (Min *et al.* 2004). This is turn negatively impacts the delicate thymic stromal compartment, which is dependent on cross-talk interactions with thymic progenitors for its sustained survival and morphogenesis (Shores *et al.* 1991; Hollander *et al.* 1995; van Ewijk *et al.* 2000; Dudakov *et al.* 2012). In this regard, two major points can be concluded based on observations made in rIL-21-treated aged mice. First, a report by Ozaki *et al*. previously demonstrated that IL-21 overexpression in WT young mice expands $LSK^+$ cells *in vivo* (Ozaki *et*

*al.* 2006). Such effect is certainly beneficial as it can increase the pool of BM progenitor cells available for thymic migration. Although we did not directly assess the level of thymus-migrating BM progenitors, we confirmed that all $LSK^+$ sub-populations in aging mice express IL-21R but fail to proliferate in response to rIL-21 administration (Fig S6). Such apparent discrepancy between our observations and those reported by Ozaki and colleagues may be explained by the lower dose or bioavailability of rIL-21 in comparison to *in vivo* overexpression. However, aged-related changes in the functional properties of $LSK^+$ cells should also be taken into account. While the frequency of LT-HSCs increases drastically in the BM of aging mice, a decrease in the ST-HSC and the transiently reconstituting MPP populations occurs in parallel (confirmed in Fig. S2) (Rossi *et al.* 2005). Such alterations are elemental to thymopoiesis as the ST-HSC and MPP fractions both lie upstream of CLP and contribute to lymphoid reconstitution (Rossi *et al.* 2005). These observations are consistent with microarray analyses revealing the existence of systematic down-regulation of LT-HSC-specific genes mediating lymphoid specification and function with aging (Rossi *et al.* 2005). This implies that rIL-21 can compensate for deficiencies affecting BM-derived progenitors by stimulating the expansion of progenitor cells such as ETPs that have already seeded the thymic compartment. Second, the increase in the absolute number of cTEC and mTEC sub-populations in rIL-21-treated aged mice cannot be mediated by a direct action of rIL-21 due to the absence of IL-21R on TECs cell surface (Rafei *et al.* 2013a). The simplest interpretation of these results is that increased thymocyte numbers enhanced lympho-stromal interactions consequently promoting TECs expansion (Hollander *et al.* 1995; van Ewijk *et al.* 2000). Indeed, BM transplantation studies using $RAG^{null}$ mice as recipients revealed a central role played by developing thymocytes in the functional organization of thymic microenvironments (van Ewijk *et al.* 2000). By first providing

signals to cTECs, DN thymocytes initiate the creation of a functional 3D organized cortical microenvironment. Such re-structuring facilitates the differentiation of DN thymocytes to DP and SP stages, which ends-up improving the survival/expansion of the mTEC compartment (van Ewijk *et al.* 2000). This is certainly a plausible explanation as the adult thymus contains non-senescent progenitor stromal cells believed to be involved in TEC maintenance (Dumont-Lagace *et al.* 2014). Thus, an involuted stroma possibly retains the capacity to regenerate in response to increased progenitor numbers. Under such context, the lack of rIL-21-induced thymopoiesis in younger mice may not be surprising as both thymic size and function are optimal at that age.

The concept that miR-181a act as a rheostat for TCR signaling is not novel (Li *et al.* 2007). Although expression of signaling molecules involved in TCR signaling is not influenced by age (Tamura *et al.* 2000), cumulative homeostatic proliferation is believed to promote progressive loss of miR-181a in aging naïve T cells overtime (Li *et al.* 2012). Only a continuous stream of newly developed T cells capable of replenishing the peripheral T-cell compartment can compensate for such intrinsic defect. In accordance with these studies, we detected lower expression of miR-181a in T cells of aged mice compared to their younger counterparts, which was further reflected on their poor TCR responsiveness (Fig. 8). However, *in vivo* provision of exogenous rIL-21 reversed the miR-181a decrease through enhanced *de novo* generation of RTEs that have incorporated the peripheral compartment. RTE maturation thereafter led to an increase in mature naïve T cells, which were competent enough to effectively respond to Trp2 vaccination and trigger a substantial delay in B16 tumor growth (Figs 7, 9, and 10). There are however data indicating that RTEs generated in aged hosts retain some intrinsic defects following TCR cross-linking (Clise-Dwyer *et al.* 2007). If so, how can we interpret the improved

effector functions observed in the rejuvenated T-cell pool of rIL-21-treated mice? Clise-Dwyer *et al*. demonstrated that RTEs generated either following transplantation of younger hosts with aged BM or after antibody-mediated depletion of peripheral T-cell in aging hosts exhibit normal effector function in comparison to RTEs developed in untreated aged animals (Clise-Dwyer *et al.* 2007). The authors explained this conundrum by suggesting that under lymphopenic conditions, levels of IL-7 are either elevated or competition for IL-7 is reduced resulting in rescued T-cell development. As stromal cells are the main producers of IL-7 in the thymus, an aging thymic microenvironment with increased TECs turnover would indeed compromise RTEs number, quality and function due to limited IL-7 availability. This highlight one of the distinctive thymopoiesis-stimulating properties of rIL-21 as it can raise the level of intrathymic IL-7 production (Fig. S5E) most likely by indirectly mediating the expansion of the stromal compartment through enhanced thymocyte proliferation therefore resulting in the generation of defects-free RTEs.

Besides physiological aging, contraction of the TCR repertoire is commonly observed in patients suffering from HIV infection, various cancers or following BM transplantation (van den Brink *et al.* 2004). There are currently no effective therapies capable of exerting a positive impact on broadening the spectrum of TCR. Studies involving IL-21R$^{-/-}$ mice clearly showed that IL-21 is dispensable for immune cell development as normal proportions of lymphocytes, monocytes, and granulocytes have been reported (Spolski & Leonard 2014). Our data nevertheless suggest that rIL-21 administration to aging hosts could have potent clinical uses related to its ability to promote the expansion of thymic progenitor cells, which can be further enhanced if combined with other thymostimulatory compounds.

## 2.4 - EXPERIMENTAL PROCEDURES

### Cell line and mice

The syngeneic C57BL/6-derived B16F0 (B16) mouse melanoma cell line was kindly provided by Dr. J. Galipeau (Atlanta, GA, USA). The RAG2p-GFP transgenic mice were kindly provided by Dr. M. Nussenzweig (Rockefeller University, NY, USA). Female WT C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). To generate IL-21R$^{-/-}$ C57BL/6 mice, commercially available sperm was purchased from the MMRRC Repository and used to fertilize female WT C57BL/6 mice. All mice were housed at the Institute for Research in Immunology and Cancer (IRIC) animal facility under specific pathogen-free conditions. All animal protocols were approved by the Animal Care Committee of Université de Montréal.

### Antibodies, cytokines and reagents

Mouse rIL-21 and granulocyte macrophage-colony stimulating factor (rGM-CSF) were purchased from Peprotech (Rocky Hill, NJ, USA). All antibodies used in flow-cytometry and Cytofix/Cytoperm Kits used for intracellular staining were purchased from BD Pharmingen (San Diego, CA, USA). The anti-SHP-2, PTPN-22, DUSP5/6 and β-actin antibodies used in western-blotting were purchased from Abcam (Cambridge, MA, USA). Quantikine ELISAs for IL-2, IL-7, IL-17 and IFNγ were purchased from R&D System (Minneapolis, MN). The CD3-CD28 beads, cell trace, and Trizol were purchased from Invitrogen (Burlington, ON, CA). The cell LyticM buffer and lipopolysaccharide (LPS) were purchased from Sigma (St-Louis, MO, USA). T-cell enrichment kits were purchased from StemCell Technologies (Vancouver, BC, Canada). The SIINFEKL and SVYDFFVWL peptides were synthesized by GenScript (Piscataway, NJ).

**Administration of rIL-21 and T-cell analyses**

For IL-21 dosage establishment, WT young (2M) or aged (15M) female mice were IP-injected with various doses of rIL-21 (12, 25, 50 or 100ug/kg of weight) in 200 ul PBS on a daily interval (total of 5 injections) in the first week. Control mice received equal volumes of PBS. Following dosage identification, all subsequent *in vivo* experiments were performed using rIL-21 at 50ug/kg. Peripheral T-cell absolute numbers were obtained by combining the analysis of blood samples collected from treated RAG2p-GFP treated mice using the Scil-Vet $ABC^{+}$ hematological analyzer (to obtain absolute counts of lymphocytes) and flow-cytometry (to obtain percentages of specific sub-populations).

**Enrichment of primary TECs**

Primary TECs were enriched from thymic lobes of WT C57Bl/6 mice as previously described (Gray *et al.* 2006).

**Western-blots**

Splenocytes were first isolated from treated mice then used to sort total $CD3^{+}$ T cells. Whole T-cell extracts were then obtained through lysis using the Cell Lytic M reagent (Sigma). Extracts where separated by electrophoresis, transferred onto Hybond-ECL membrane, and probed using primary antibodies. Densitometry analysis was conducted by first imaging the chemiluminescent blots with the ImageQuant LAS 4000 imager (GE Healthcare Life Sciences) followed by analysis using the ImageQuant TL software (GE Healthcare Life Sciences).

**Expression analysis of miR-181a**

T cells were first sorted directly in 900 µl Trizol ($10^6$ cells per tube), lysed followed by RNA was extraction in Trizol reagent (Invitrogen). RNA purification was further carried out using the RNA extraction kit (QIAgen). Reverse transcription was performed using the High Capacity cDNA reverse transcription kit and qPCR was performed with a 7900HT Fast Real-Time PCR system at IRIC's genomic core facility. Target gene values were normalized to endogenous control *Gapdh*.

**Intracellular staining**

For analysis of phosphorylated (p)Lck, ZAP-70, and ERK, splenocytes were first isolated from treated animals prior to *in vitro* stimulation using CD3/CD28 dynabeads (Invitrogen) for 5 min. Splenocytes were then washed then surface stained for CD4 and CD8 prior to intracellular staining using PE-labelled primary antibodies according to manufacturer's instructions.

**Histological analyses**

Chosen organs were harvested from treated mice, fixed in 10% formalin before mounting in paraffin. Sections were then stained with hematoxylin and eosin then scanned using the NanoZoomer Digital Pathology system and NPD.scan 2.3.4 software (Hamamatsu).

**DC vaccination and tumor challenge**

To generate DCs for vaccination, BM cells were extracted from tibia and femur bones of 8 weeks old male mice and plated in 10 cm non-tissue culture-treated Petri dishes in 10 mL of

complete RPMI 1640 medium (0.048 mmol/L β-mercaptoethanol, 2 mmol/L L-glutamine, 10% fetal bovine serum, 2 mmol/L penicillin-streptavidin) supplemented with 10 ng/mL rGM-CSF (Peprotech). The media was changed at days 3 and 6 of culture. To induce DC maturation, 1 μg/mL LPS (Sigma) was added at day 8 for 24 hours. Following confirmation of a mature phenotype (>80% of adherent cells were $CD80^+CD86^+MHCI^+MHCII^+$), DCs were pulsed with OVA- or Trp2-derived peptides (GeneScript) for 4 hours then harvested for intravenous vaccination three weeks following the last cytokine or PBS treatment. Two weeks following the last DC injection, vaccinated C57BL/6 mice were SC-challenged with $5\times10^5$ syngeneic B16 tumor cells. Tumor volume was measured as [(length × $width^2$)/2], and mice were sacrificed when the tumor volume reached 2000 $mm^3$ or if it ulcerated.

**Proliferation and cytokine measurements**

For all assays conducted using non-vaccinated mice, total or fractionated $CD4^+$ and $CD8^+$ T cells were first isolated from individual mice by negative selection then plated at $10^5$ cells/well in 96-well plates. To trigger proliferation or activation, T cells were stimulated with CD3/CD28 dynabeads in a 1:1 ratio. For *in vitro* re-call responses following vaccination, splenocytes were pulsed with 1ug/ml Trp-2 peptide. In all conditions, cells or supernatants were collected 48hrs post-stimulation for further analyses.

**Statistical analyses**

*P*-values were calculated using the ANOVA and Log-Rank statistical test where applicable. Log-rank testing was performed using software available at the Walter and Eliza Hall Institute Web site (http://bioinf.wehi.edu.au/software/russell/logrank/).

## 2.5 - ACKNOWLEDGMENTS

We are grateful to Danièle Gagné and Serge Sénéchal (for sorting and flow cytometry assistance), Raphaëlle Lambert (for qPCR studies) and to the staff of the IRIC histology and animal care facility for their assistance. This work was supported by a Cancer Research Society (CRS) operating grant. Moutih Rafei holds a Fonds de la Recherche en Santé du Québec Junior 1 award.

## 2.6 - AUTHOR CONTRIBUTIONS

EAC designed most of the study, carried out experiments, analyzed the data, prepared the figures and wrote the first draft of the manuscript. AT, SP, RK and SZ performed some experiments and contributed to data analysis and manuscript preparation. MR designed the study, discussed the results with all authors and wrote the manuscript. All authors approved the final version of manuscript. The authors declare that they have no conflict of interest.

## 2.7 – REFERENCES


Ahmed M, Lanzer KG, Yager EJ, Adams PS, Johnson LL, Blackman MA (2009). Clonal expansions and loss of receptor diversity in the naive CD8 T cell repertoire of aged mice. *Journal of immunology (Baltimore, Md. : 1950)*. **182**, 784-792.

Attridge K, Wang CJ, Wardzinski L, Kenefeck R, Chamberlain JL, Manzotti C, Kopf M, Walker LS (2012). IL-21 inhibits T cell IL-2 production and impairs Treg homeostasis. *Blood*. **119**, 4656-4664.

Berkley AM, Hendricks DW, Simmons KB, Fink PJ (2013). Recent thymic emigrants and mature naive T cells exhibit differential DNA methylation at key cytokine loci. *Journal of immunology (Baltimore, Md. : 1950)*. **190**, 6180-6186.

Boehm T, Swann JB (2013). Thymus involution and regeneration: two sides of the same coin? *Nature reviews. Immunology*. **13**, 831-838.

Boursalian TE, Golob J, Soper DM, Cooper CJ, Fink PJ (2004). Continued maturation of thymic emigrants in the periphery. *Nature immunology*. **5**, 418-425.

Clise-Dwyer K, Huston GE, Buck AL, Duso DK, Swain SL (2007). Environmental and intrinsic factors lead to antigen unresponsiveness in CD4(+) recent thymic emigrants from aged mice. *Journal of immunology (Baltimore, Md. : 1950)*. **178**, 1321-1331.

Decman V, Laidlaw BJ, Doering TA, Leng J, Ertl HC, Goldstein DR, Wherry EJ (2012). Defective CD8 T cell responses in aged mice are due to quantitative and qualitative changes in virus-specific precursors. *Journal of immunology (Baltimore, Md. : 1950)*. **188**, 1933-1941.

Dudakov JA, Hanash AM, Jenq RR, Young LF, Ghosh A, Singer NV, West ML, Smith OM, Holland AM, Tsai JJ, Boyd RL, van den Brink MR (2012). Interleukin-22 drives endogenous thymic regeneration in mice. *Science (New York, N.Y.)*. **336**, 91-95.

Dumont-Lagace M, Brochu S, St-Pierre C, Perreault C (2014). Adult thymic epithelium contains nonsenescent label-retaining cells. *Journal of immunology (Baltimore, Md. : 1950)*. **192**, 2219-2226.

Eaton SM, Burns EM, Kusser K, Randall TD, Haynes L (2004). Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses. *The Journal of experimental medicine*. **200**, 1613-1622.

Garcia GG, Miller RA (1997). Differential tyrosine phosphorylation of zeta chain dimers in mouse CD4 T lymphocytes: effect of age. *Cellular immunology*. **175**, 51-57.

Garcia GG, Miller RA (2001). Single-cell analyses reveal two defects in peptide-specific activation of naive T cells from aged mice. *Journal of immunology (Baltimore, Md. : 1950)*. **166**, 3151-3157.

Garcia GG, Miller RA (2002). Age-dependent defects in TCR-triggered cytoskeletal rearrangement in CD4+ T cells. *Journal of immunology (Baltimore, Md. : 1950)*. **169**, 5021-5027.

Gray DH, Seach N, Ueno T, Milton MK, Liston A, Lew AM, Goodnow CC, Boyd RL (2006). Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. *Blood*. **108**, 3777-3785.

Hale JS, Boursalian TE, Turk GL, Fink PJ (2006). Thymic output in aged mice. *Proceedings of the National Academy of Sciences of the United States of America*. **103**, 8447-8452.

Haynes L, Swain SL (2006). Why aging T cells fail: implications for vaccination. *Immunity*. **24**, 663-666.

Hollander GA, Wang B, Nichogiannopoulou A, Platenburg PP, van Ewijk W, Burakoff SJ, Gutierrez-Ramos JC, Terhorst C (1995). Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. *Nature*. **373**, 350-353.

Houston EG, Jr., Nechanitzky R, Fink PJ (2008). Cutting edge: Contact with secondary lymphoid organs drives postthymic T cell maturation. *Journal of immunology (Baltimore, Md. : 1950)*. **181**, 5213-5217.

Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, Oukka M, Kuchroo VK (2007). IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature*. **448**, 484-487.

Lages CS, Suffia I, Velilla PA, Huang B, Warshaw G, Hildeman DA, Belkaid Y, Chougnet C (2008). Functional regulatory T cells accumulate in aged hosts and promote chronic infectious disease reactivation. *Journal of immunology (Baltimore, Md. : 1950)*. **181**, 1835-1848.

Li G, Yu M, Lee WW, Tsang M, Krishnan E, Weyand CM, Goronzy JJ (2012). Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. *Nature medicine*. **18**, 1518-1524.

Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, Braich R, Manoharan M, Soutschek J, Skare P, Klein LO, Davis MM, Chen CZ (2007). miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell*. **129**, 147-161.

Lynch HE, Goldberg GL, Chidgey A, Van den Brink MR, Boyd R, Sempowski GD (2009). Thymic involution and immune reconstitution. *Trends in immunology*. **30**, 366-373.

Maue AC, Yager EJ, Swain SL, Woodland DL, Blackman MA, Haynes L (2009). T-cell immunosenescence: lessons learned from mouse models of aging. *Trends in immunology*. **30**, 301-305.

Min H, Montecino-Rodriguez E, Dorshkind K (2004). Reduction in the developmental potential of intrathymic T cell progenitors with age. *Journal of immunology (Baltimore, Md. : 1950)*. **173**, 245-250.

Monroe RJ, Seidl KJ, Gaertner F, Han S, Chen F, Sekiguchi J, Wang J, Ferrini R, Davidson L, Kelsoe G, Alt FW (1999). RAG2:GFP knockin mice reveal novel aspects of RAG2 expression in primary and peripheral lymphoid tissues. *Immunity*. **11**, 201-212.

Ozaki K, Hishiya A, Hatanaka K, Nakajima H, Wang G, Hwu P, Kitamura T, Ozawa K, Leonard WJ, Nosaka T (2006). Overexpression of interleukin 21 induces expansion of hematopoietic progenitor cells. *International journal of hematology*. **84**, 224-230.

Parkhurst MR, Fitzgerald EB, Southwood S, Sette A, Rosenberg SA, Kawakami Y (1998). Identification of a shared HLA-A*0201-restricted T-cell epitope from the melanoma antigen tyrosinase-related protein 2 (TRP2). *Cancer research*. **58**, 4895-4901.

Peluso I, Fantini MC, Fina D, Caruso R, Boirivant M, MacDonald TT, Pallone F, Monteleone G (2007). IL-21 counteracts the regulatory T cell-mediated suppression of human CD4+ T lymphocytes. *Journal of immunology (Baltimore, Md. : 1950)*. **178**, 732-739.

Rafei M, Dumont-Lagace M, Rouette A, Perreault C (2013a). Interleukin-21 accelerates thymic recovery from glucocorticoid-induced atrophy. *PloS one*. **8**, e72801.

Rafei M, Hardy MP, Williams P, Vanegas JR, Forner KA, Dulude G, Labrecque N, Galipeau J, Perreault C (2011). Development and function of innate polyclonal TCRalphabeta+ CD8+ thymocytes. *Journal of immunology (Baltimore, Md. : 1950)*. **187**, 3133-3144.

Rafei M, Rouette A, Brochu S, Vanegas JR, Perreault C (2013b). Differential effects of gammac cytokines on postselection differentiation of CD8 thymocytes. *Blood*. **121**, 107-117.

Rossi DJ, Bryder D, Zahn JM, Ahlenius H, Sonu R, Wagers AJ, Weissman IL (2005). Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proceedings of the National Academy of Sciences of the United States of America*. **102**, 9194-9199.

Shores EW, Van Ewijk W, Singer A (1991). Disorganization and restoration of thymic medullary epithelial cells in T cell receptor-negative scid mice: evidence that receptor-bearing lymphocytes influence maturation of the thymic microenvironment. *European journal of immunology*. **21**, 1657-1661.

Spolski R, Leonard WJ (2014). Interleukin-21: a double-edged sword with therapeutic potential. *Nature reviews. Drug discovery*. **13**, 379-395.

Tamir A, Eisenbraun MD, Garcia GG, Miller RA (2000). Age-dependent alterations in the assembly of signal transduction complexes at the site of T cell/APC interaction. *Journal of immunology (Baltimore, Md. : 1950)*. **165**, 1243-1251.

Tamura T, Kunimatsu T, Yee ST, Igarashi O, Utsuyama M, Tanaka S, Miyazaki S, Hirokawa K, Nariuchi H (2000). Molecular mechanism of the impairment in activation signal transduction in CD4(+) T cells from old mice. *International immunology*. **12**, 1205-1215.

Tough DF, Sprent J (1994). Turnover of naive- and memory-phenotype T cells. *The Journal of experimental medicine*. **179**, 1127-1135.

Tsukamoto H, Clise-Dwyer K, Huston GE, Duso DK, Buck AL, Johnson LL, Haynes L, Swain SL (2009). Age-associated increase in lifespan of naive CD4 T cells contributes to T-cell homeostasis but facilitates development of functional defects. *Proceedings of the National Academy of Sciences of the United States of America*. **106**, 18333-18338.

Van den Brink MR, Alpdogan O, Boyd RL (2004). Strategies to enhance T-cell reconstitution in immunocompromised patients. *Nature reviews. Immunology*. **4**, 856-867.

Van Ewijk W, Hollander G, Terhorst C, Wang B (2000). Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. *Development (Cambridge, England)*. **127**, 1583-1591.

Yager EJ, Ahmed M, Lanzer K, Randall TD, Woodland DL, Blackman MA (2008). Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *The Journal of experimental medicine*. **205**, 711-723.

Yu W, Nagaoka H, Jankovic M, Misulovin Z, Suh H, Rolink A, Melchers F, Meffre E, Nussenzweig MC (1999). Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. *Nature*. **400**, 682-687.

Zanni F, Vescovini R, Biasini C, Fagnoni F, Zanlari L, Telera A, Di Pede P, Passeri G, Pedrazzoni M, Passeri M, Franceschi C, Sansoni P (2003). Marked increase with age of type 1 cytokines within memory and effector/cytotoxic CD8+ T cells in humans: a contribution to understand the relationship between inflammation and immunosenescence. *Experimental gerontology*. **38**, 981-987.

Zhang X, Fujii H, Kishimoto H, LeRoy E, Surh CD, Sprent J (2002). Aging leads to disturbed homeostasis of memory phenotype CD8(+) cells. *The Journal of experimental medicine*. **195**, 283-293.

## 2.8 - SUPPORTING INFORMATION

**FIGURE S5:** Analysis of thymic $GFP^+$ content, absolute counts and IL-7 production following rIL-21 administration.

**FIGURE S6:** Administration of rIL-21 to aged mice has no beneficial effect on the BM compartment.

**FIGURE S7:** Transcription factors quantification in T cells derived from treated mice.

**FIGURE S8:** Assessment of autoimmune signs following rIL-21 administration.

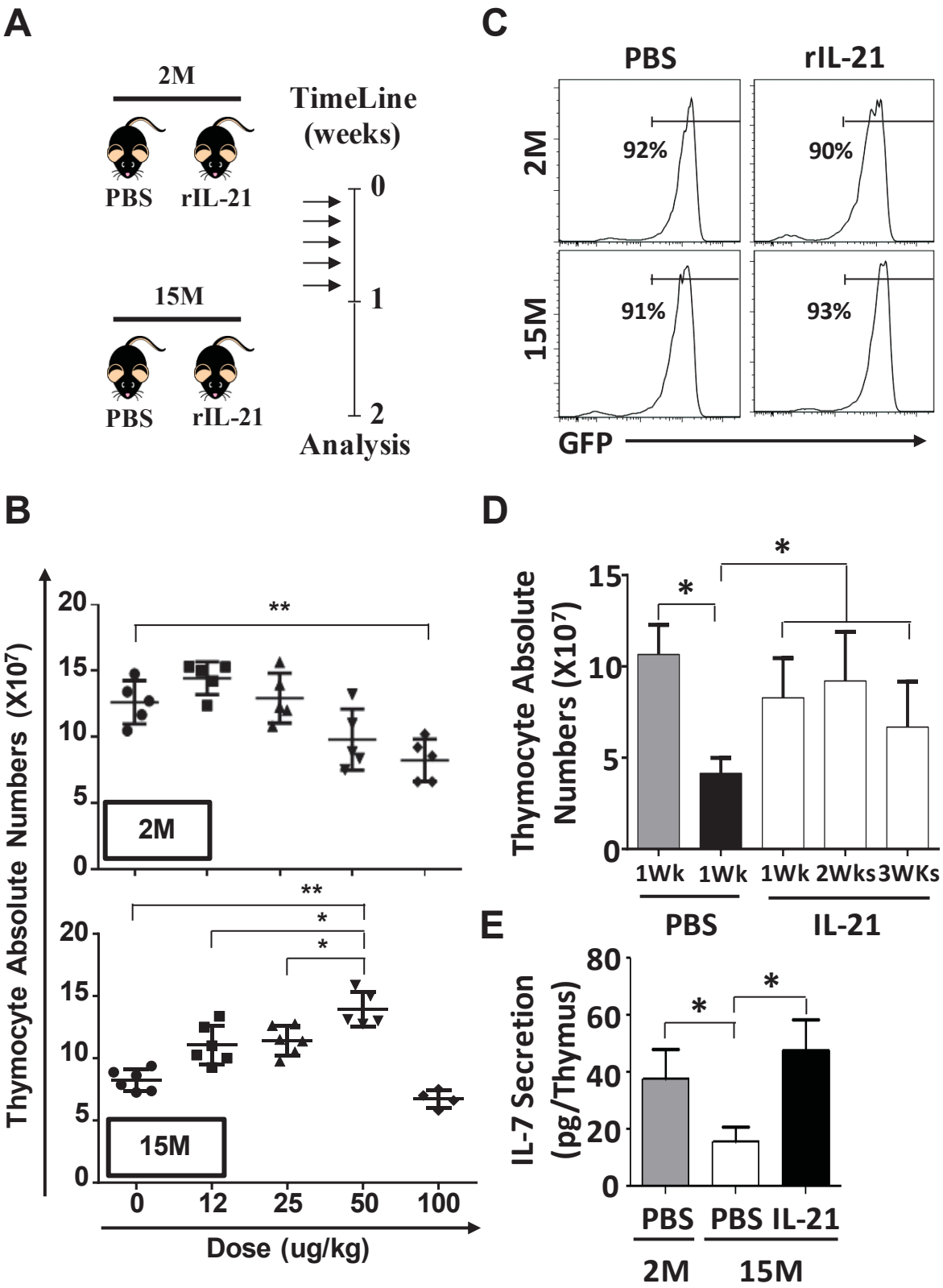
**FIGURE S9:** Generation and characterisation of BM-derived DC for vaccination.

**FIGURE S10:** rIL-21 administration to aged mice increases thymic output leading to improved immune functions.

## SUPPLEMENTARY FIGURES

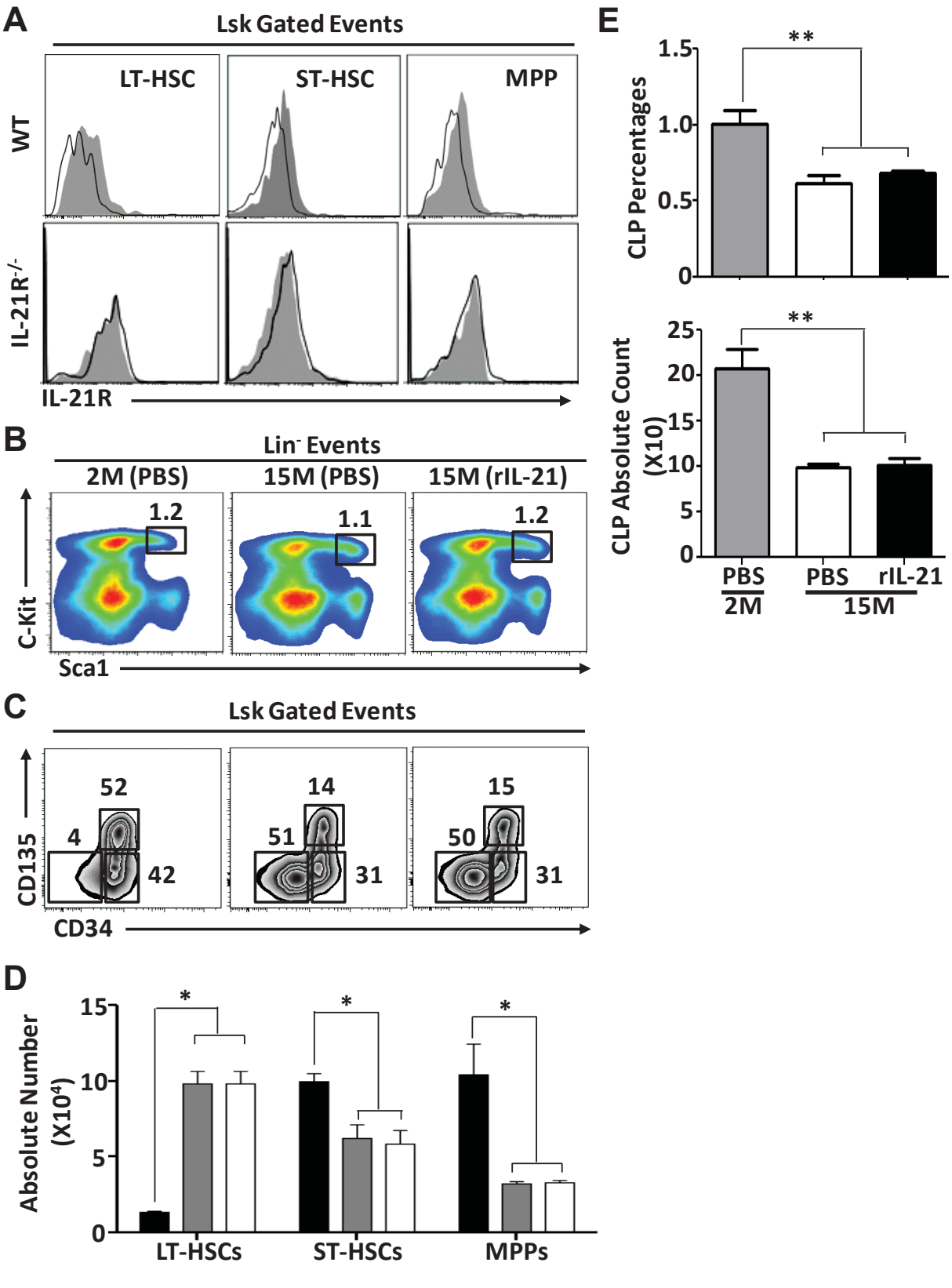
**SUPPLEMENTARY FIGURE S5: ANALYSIS OF THYMIC GFP$^+$ CONTENT, ABSOLUTE COUNTS, AND IL-7 PRODUCTION FOLLOWING RIL-21 ADMINISTRATION.**

A) Schematic diagram representing the strategy and group of mice used to identify the optimal dose of rIL-21 capable of enhancing *de novo* thymopoiesis. Thymi were collected and analyzed one week following the last rIL-21 administration. B) Total thymocyte count following administration of ascending doses of rIL-21 to young (2M) or aged (15M) WT female C57BL/6 mice. C) Representative flow-cytometry analysis of thymic GFP content. D) Absolute counts of total thymocytes at 1, 2 or 3 weeks post-treatments. All data are representative of three independent experiments (n = 5/group with *$p<0.05$).


A
2M
PBS
rIL-21
15M
PBS
rIL-21
TimeLine (weeks)
0
1
2
Analysis
C
PBS
rIL-21
2M
15M
92%
90%
91%
93%
GFP
B
Thymocyte Absolute Numbers (X10^7)
2M
15M
**
*
*
0
12
25
50
100
Dose (ug/kg)
D
Thymocyte Absolute Numbers (X10^7)
*
*
1Wk
1Wk
1Wk
2Wks
3WKs
PBS
IL-21
E
IL-7 Secretion (pg/Thymus)
*
*
PBS
PBS
IL-21
2M
15M

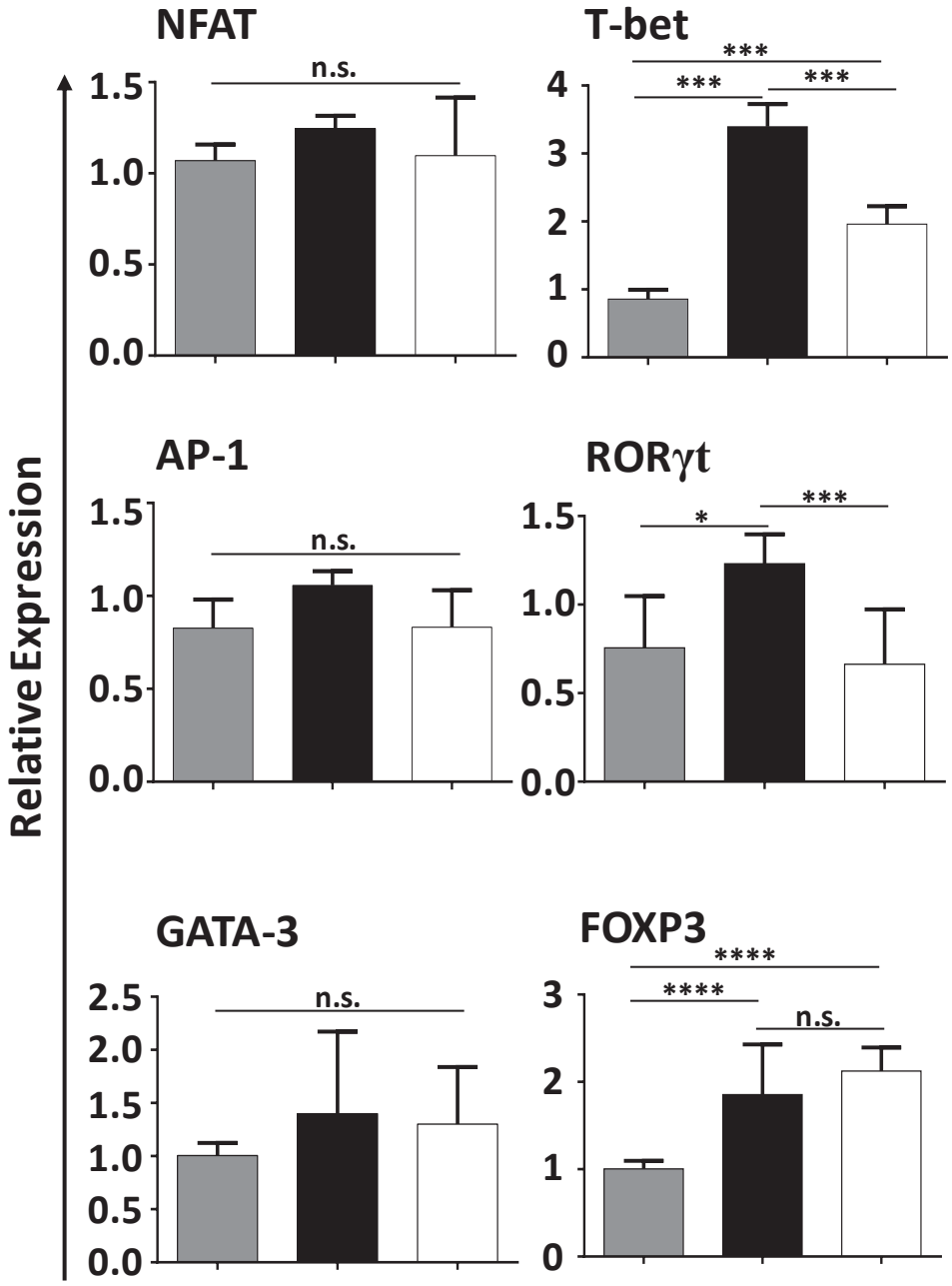
**SUPPLEMENTARY FIGURE S6: ADMINISTRATION OF RIL-21 TO AGED MICE HAS NO BENEFICIAL EFFECT ON THE BM COMPARTMENT.**

A) Representative flow-cytometry analysis of IL-21R expression on the surface of LT-, ST-HSCs and MPPs derived from WT (top panel) or IL-21R$^{-/-}$ mice (lower panel). Filled histograms represent IL-21R expression. B-C) Percentages of total LSK$^{+}$ cells or LT-, ST-HSCs as well as MPPs as depicted by flow-cytometry. D) Absolute counts of LSK$^{+}$ sub-populations. e) CLP percentage and absolute counts in 2M (PBS□), 15M (PBS□), and 15M (rIL-21■) aged mice. All data are representative of three independent experiments (n=5/group).


A
Lsk Gated Events
LT-HSC
ST-HSC
MPP
WT
IL-21R-/-
IL-21R
B
Lin- Events
2M (PBS)
15M (PBS)
15M (rIL-21)
1.2
1.1
1.2
C-Kit
Sca1
C
Lsk Gated Events
52
4
42
14
51
31
15
50
31
CD135
CD34
D
Absolute Number (X10⁴)
15
10
5
0
*
*
*
LT-HSCs
ST-HSCs
MPPs
E
**
1.5
1.0
0.5
0
CLP Percentages
**
25
20
15
10
5
0
CLP Absolute Count (X10)
PBS
PBS
rIL-21
2M
15M

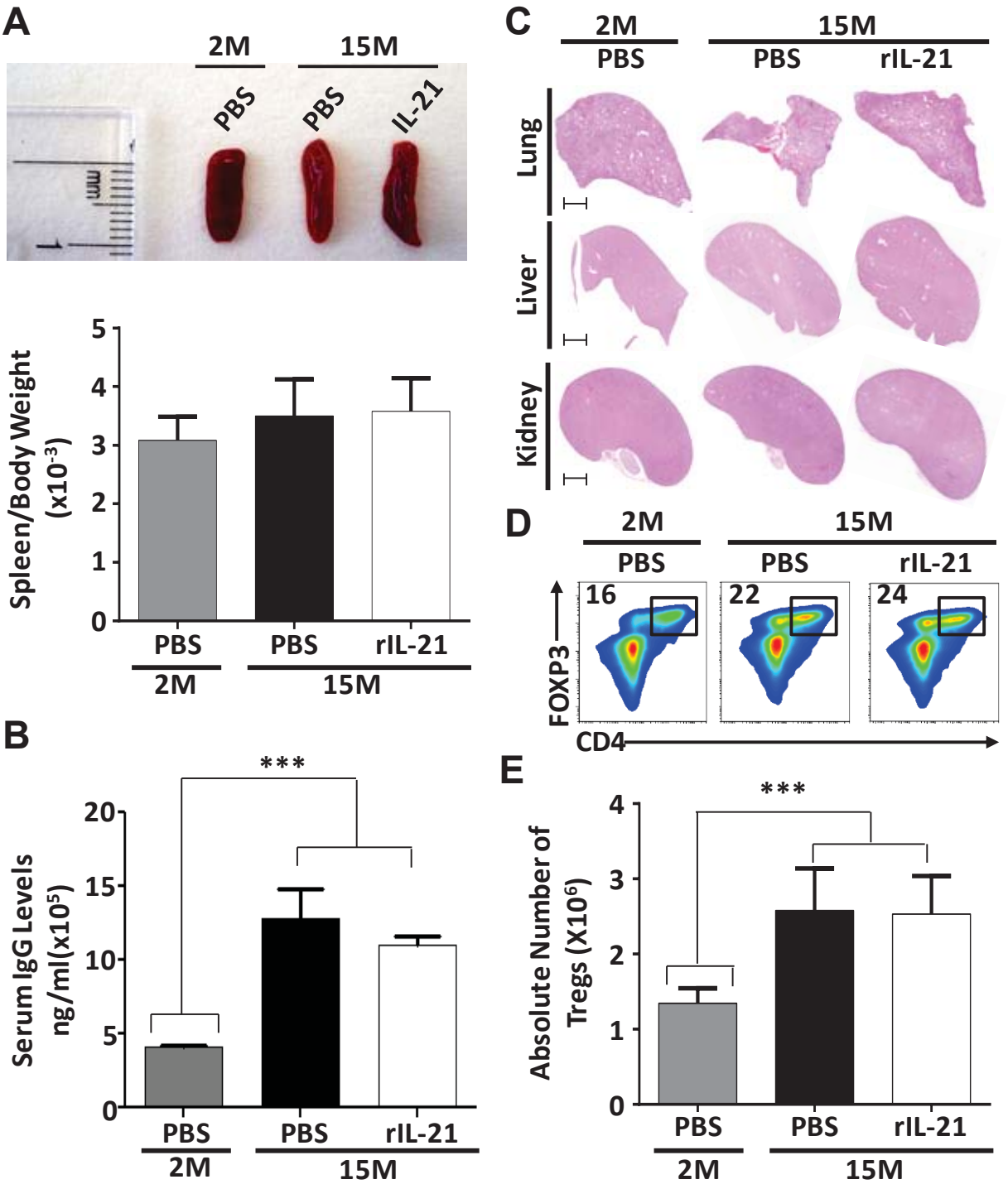
**SUPPLEMENTARY FIGURE S7: TRANSCRIPTION FACTORS QUANTIFICATION IN T CELLS DERIVED FROM TREATED MICE.**

T cells derived from treated mice were isolated then lysed to extract total mRNA. qPCR analyses were then conducted to quantify the expression of targeted genes relative to endogenous controls. All data are representative of three independent experiments (n=5/group with *$p<0.05$, ***$p<0.001$, and ****$p<0.0001$).


NFAT
n.s.
1.5
1.0
0.5
0.0
T-bet
***
***
***
4
3
2
1
0
AP-1
n.s.
1.5
1.0
0.5
0.0
RORγt
*
***
1.5
1.0
0.5
0.0
GATA-3
n.s.
2.5
2.0
1.5
1.0
0.5
0.0
FOXP3
****
****
n.s.
3
2
1
0
Relative Expression

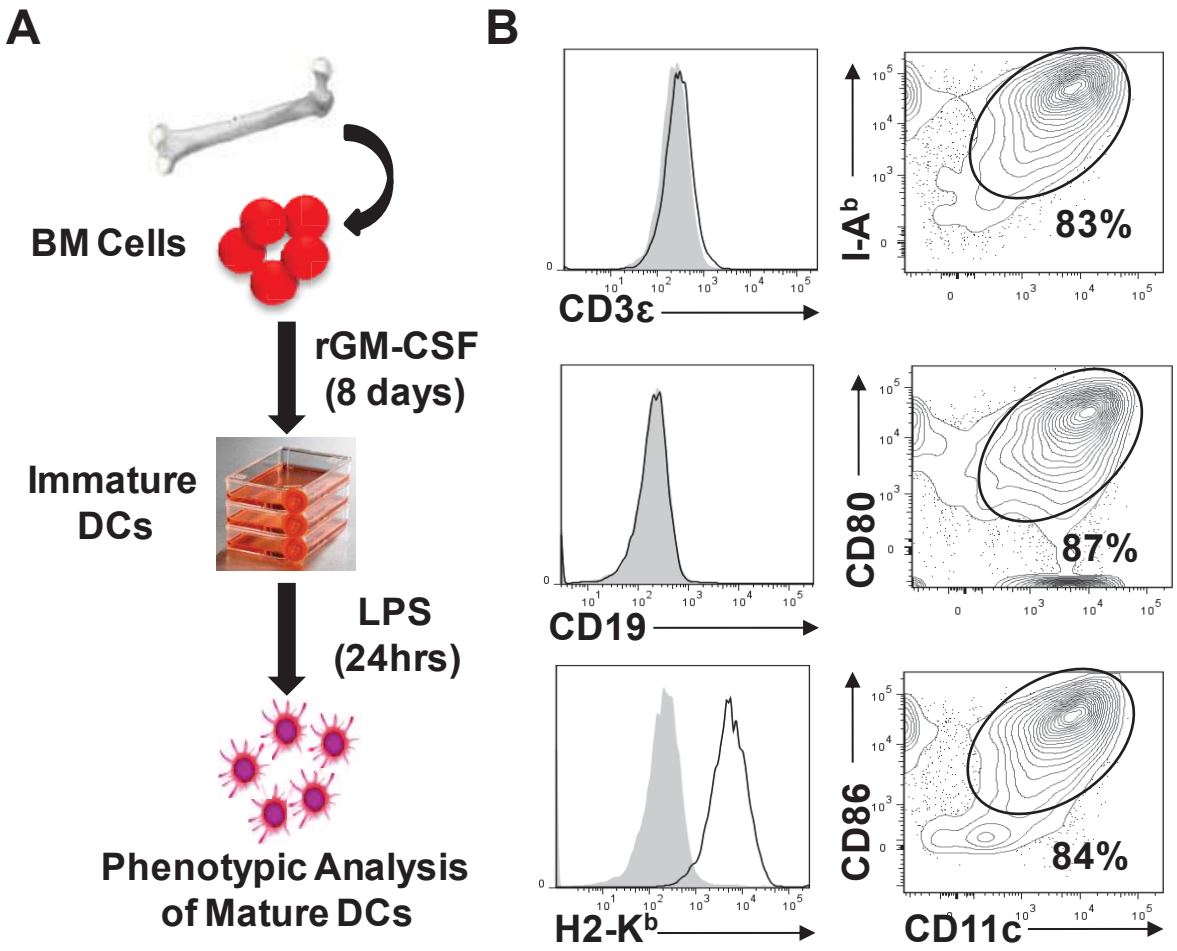
**SUPPLEMENTARY FIGURE S8: ASSESSMENT OF AUTOIMMUNE SIGNS FOLLOWING RIL-21 ADMINISTRATION.**

A) A representative photograph of spleens and their relative spleen to body weight derived from treated mice. B) Serum IgG levels quantified by ELISA. C) Hematoxylin-eosin staining of formalin-fixed sections of lung, liver and kidney of young, PBS- or rIL-21-treated aged mice. Scale bars represent 2 mm. D) Representative flow-cytometry analysis of $CD4^+$ $T_{regs}$ in all experimental groups. We gated on $CD25^+CD4^+$ T cells prior to FOXP3 intracellular analysis. E) Quantification of $CD4^+$ $T_{regs}$ according to flow-cytometry analysis and splenocyte counts. All data are representative of three independent experiments (n = 5/group with ***$p<0.001$).


A
2M
15M
PBS
PBS
IL-21
Spleen/Body Weight (x10-3)
PBS
PBS
rIL-21
2M
15M
B
***
Serum IgG Levels ng/ml(x10^5)
PBS
PBS
rIL-21
2M
15M
C
2M
15M
PBS
PBS
rIL-21
Lung
Liver
Kidney
D
2M
15M
PBS
PBS
rIL-21
16
22
24
FOXP3
CD4
E
***
Absolute Number of Tregs (X10^6)
PBS
PBS
rIL-21
2M
15M

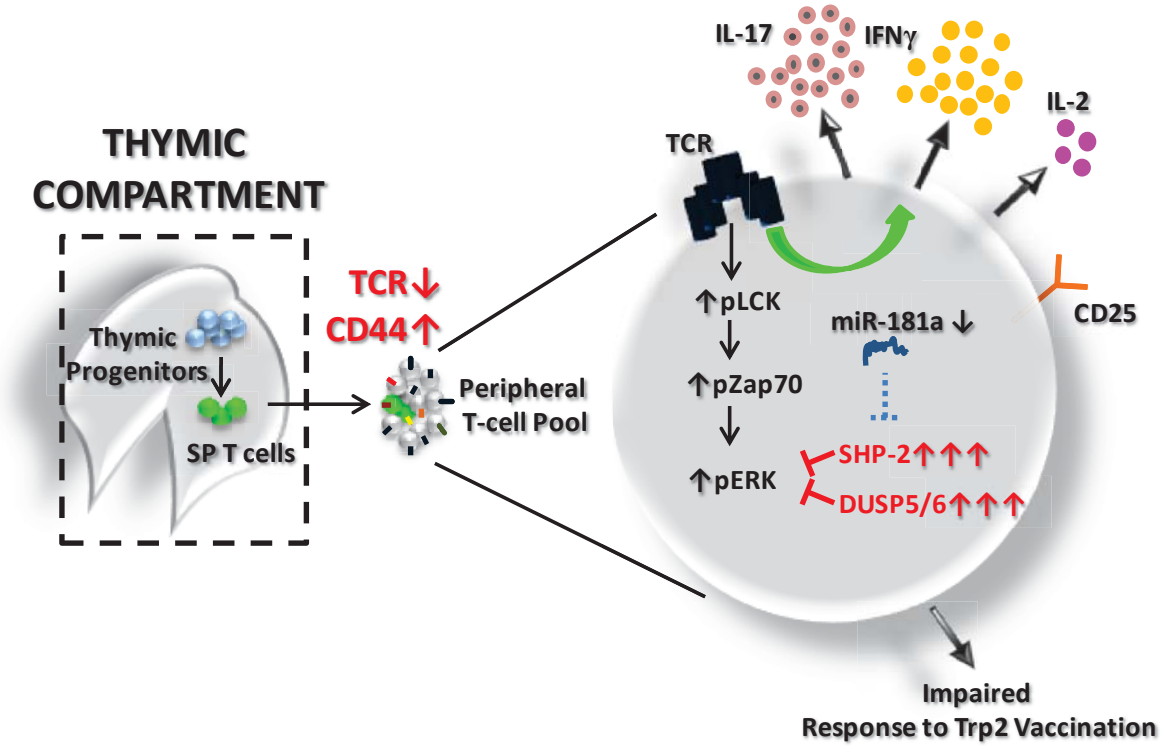
**SUPPLEMENTARY FIGURE S9: GENERATION AND CHARACTERISATION OF BM-DERIVED DC FOR VACCINATION.**

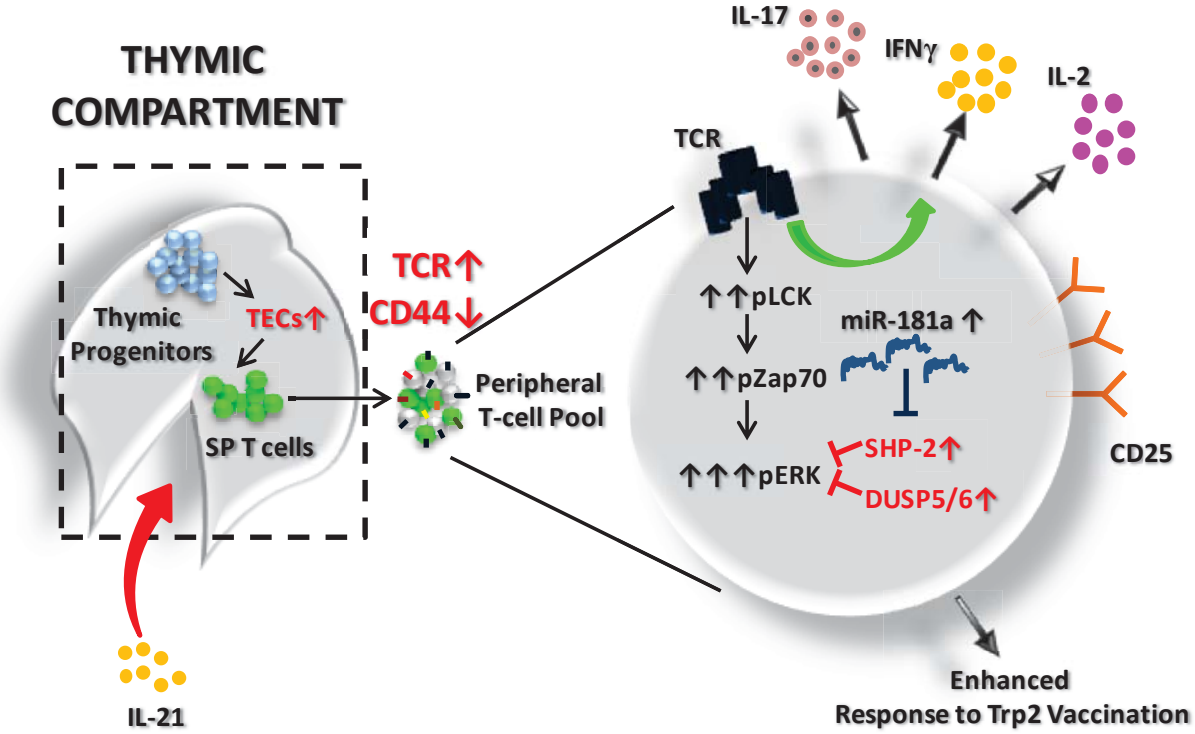
A) To generate BM-derived DCs, femur and tibias of male C57BL/6 mice where flushed to collect total nucleated cells, which were then plated for 8 days with rGM-CSF (10ng/ml). LPS was added to stimulate DC maturation at day 9. B) Representative flow-cytometry analysis of mature DC phenotype (>80% of DCs were $CD11c^{+}CD80^{+}CD86^{+}H2\text{-}K^{b+}I\text{-}A^{b+}$) using all non-adherent cells collected at day 10.


A
BM Cells
rGM-CSF
(8 days)
Immature
DCs
LPS
(24hrs)
Phenotypic Analysis
of Mature DCs
B
CD3ε
I-Ab
83%
CD19
CD80
87%
H2-Kb
CD86
84%
CD11c

**SUPPLEMENTARY FIGURE S10: RIL-21 ADMINISTRATION TO AGED MICE INCREASES THYMIC OUTPUT LEADING TO IMPROVED IMMUNE FUNCTIONS.**

In aging mice, thymopoiesis is diminished causing attrition of the TCR repertoire and accumulation of peripheral $CD44^{hi}$ T cells. In parallel, increased post-thymic life span leads to accumulated SHP-2 and DUSP5/6 due to decreased expression of miR-181a. As such, TCR stimulation of aging naïve T cells is weakened leading to lower CD25 expression along with poor secretion of IL-2 and thus, impaired immune functions. When given to aged mice, rIL-21 promotes thymopoiesis leading to increased T-cell output and improved TCR diversity. As RTEs increase the proportion of naïve T cells expressing higher level of miR-181a, SHP-2 and DUSP5/6 levels diminish allowing better T-cell responses translating into potent immunity.


THYMIC COMPARTMENT
Thymic Progenitors
SP T cells
TCR↓
CD44↑
Peripheral T-cell Pool
IL-17
IFNγ
IL-2
TCR
↑pLCK
↑pZap70
↑pERK
miR-181a ↓
SHP-2↑↑↑
DUSP5/6↑↑↑
CD25
Impaired Response to Trp2 Vaccination


THYMIC COMPARTMENT
Thymic Progenitors
TECs↑
SP T cells
IL-21
TCR↑
CD44↓
Peripheral T-cell Pool
IL-17
IFNγ
IL-2
TCR
↑↑pLCK
↑↑pZap70
↑↑↑pERK
miR-181a ↑
SHP-2↑
DUSP5/6↑
CD25
Enhanced Response to Trp2 Vaccination

# CHAPTRE 3

## *GENERAL CONCLUSION*

## 3.0 – Conclusion

Stimulating the immune system to protect the host from infectious diseases has been a triumphal area of research as of the late 18th century. Since then, scientists believed in extending this success to generate protective cancer vaccines. Unfortunately however, this strategy was quickly found not to suit the elderly population due to their decline in T-cell output caused by thymic regression and accumulation of aged lymphocytes endowed with stimulation-related defects. Thus, two fundamental questions remain to be addressed: should available cancer vaccines be re-engineered or should the elderly immunity be enhanced? The common denominator of all compiled data suggests that an effective cancer vaccination strategy requires the sustained thymic development of young and sensitive T cells capable of recognizing a panel of diversified targets.

Although several vaccine-supportive therapies were explored, their administration was either ineffective, had transient effects or triggered division of aged endogenous circulating T cells rather than generation of new ones in the thymus. These include: i) administration of exogenous growth factors and cytokines, such as KGF, growth hormone, GRL, IL-7, IL-15, insulin-like growth factor-1, IL-12, or Flt3L; ii) adoptive transfer of T-cell precursors generated *in vitro*; or iii) the chemical or surgical ablation of sex steroids (**FIGURE 11**) (57). While most of these treatments primarily promote thymopoiesis directly, IL-22, KGF and SSA enhance thymic regeneration by acting on thymic stromal cells (48-36-24). Although adoptive transfer of *in vitro* generated T-cell precursors may aid in thymic regeneration, the mechanism behind its impact on the thymus is poorly understood but may imply improved intrathymic lympho-stromal interactions.

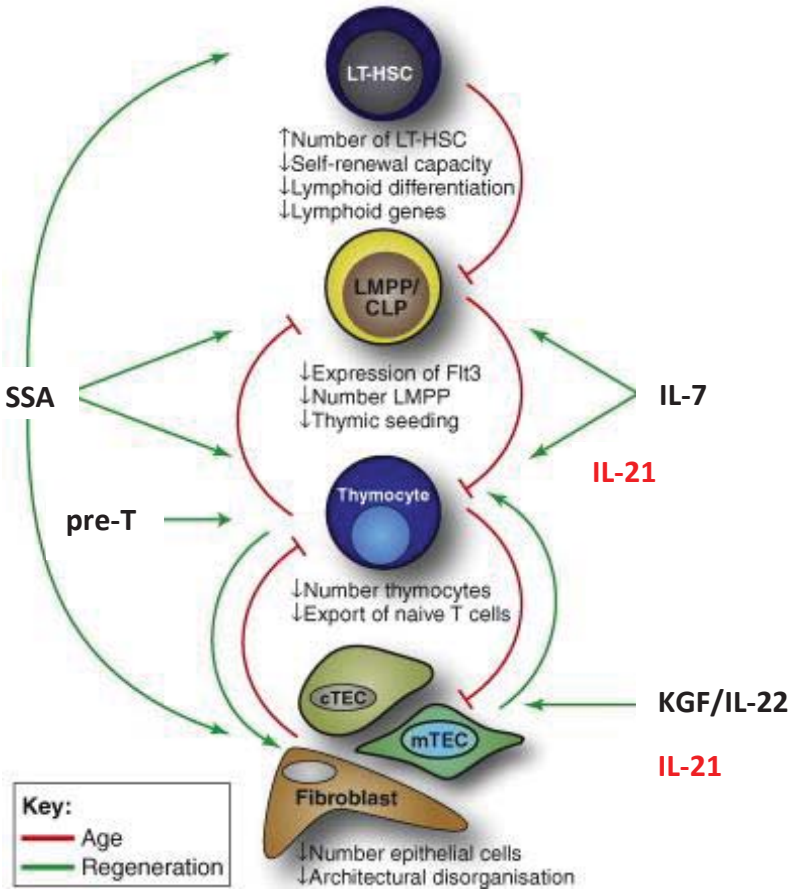



**FIGURE 11: THE MOST PROMISING STRATEGIES DEVELOPED FOR REGULATION OF LYMPHOPOIESIS.**

Overview of several therapeutic approaches and their cognate targeted compartments (**Dudakov JA *et al*. Trends in Immunology 2010)** (54).

On top of previously developed strategy, we show in the present study that administration of IL-21 to old mice stimulate thymopoiesis *de novo* leading to the generation of naïve T cells as depicted with increased accumulation of RTEs in the periphery using the RAG2-GFP model. Nonetheless, the observed effects were not limited on hematopoietic cells as TEC numbers were also improved. As a result, a highly diversified TCR repertoire was restored along with the appearance of a rejuvenated T-cell pool displaying improved TCR sensitivity and T-cell functionality (enhanced proliferation, IL-2 production and CD25 expression). Finally, we have shown that old mice treated with IL-21 display delayed tumor growth following Trp-2 vaccination. Collectively, our results indicate that IL-21 acts directly on thymic progenitors (ETP, DN, DP thymocytes) with a significant but indirect impact on the stromal compartment (**FIGURE 11**).

An NH2 terminal methionylated form of bacterially-expressed recombinant IL-21 (manufactured by ZymoGenetics, Inc.) was granted an orphan drug status by the FDA in 2005 for the treatment of patients with advanced or aggressive melanoma (59). Since then, more than a dozen clinical trials were conducted (clinicaltrials.gov) and revealed that IL-21 was shown to display antitumor activity in both renal cell carcinoma (RCC) and metastatic melanoma with partial response achieved in treated patients. Based on these outcomes, the therapeutic effect of IL-21 was sought to be enhanced if combined with other immunostimulatory agents. This hypothesis stems from preclinical studies showing significant antitumor effect using an in vivo model of RCC with the combination of IL-21 and IFN-α (60). A second study confirmed this notion by demonstrating that IL-21 combined with low-dose IL-2 in a melanoma mouse model substantially enhances the

tumor free survival compared to either agents alone (61). Although the primary objective of these trials was not focused on thymopoiesis, IL-21 has been reported to increase the frequency of highly responsive human naïve (CD62L+CD45RA+) T cells in circulation (60). As such, our findings will strengthen our understanding on the role played by IL-21 in thymopoiesis. Consequently, significant ramifications on the future use of IL-21 will emerge in the context of preventive/therapeutic measures aimed at enhancing immunity of the aging population against cancer and infectious diseases.

## BIBLIOGRAPHY


1. Pancer Z, Cooper MD (2006). The evolution of adaptive immunity. *Annual Review of Immunology* 24 (1): 497–518.
2. CA Janeway (2001). *Immunobiology:* The Immune System in Health and Disease. 5th edition.
3. Thomas Boehm (2011). Design principles of adaptive immune systems. *Nature reviews*. *Immunology*. 10.1038.
4. Hans-Reimer Rodewald (2008). Thymus Organogenesis. *Annual Review of Immunology*. 10.1146.
5. Lynch HE, *et al*. (2009). Thymic involution and immune reconstitution. *Trends in Immunology*. 10.1016.
6. Danielle Aw, Donald B.Palmer (2011). The Origin and Implication of Thymic Involution. *Aging and Disease*. 2152-5250.
7. Gail Pearse (2006) Normal Structure, Function and Histology of the Thymus. *Toxicologic Pathology*, 34:504–514.
8. Graham Anderson, and Yousuke Takaham (2012). Thymic epithelial cells: working class heroes for T cell development and repertoire selection. *Trends in Immunology*. 10.1016.
9. C. Clare Blackburn and Nancy R Manley (2004). Developing a new paradigm for thymus organogenesis. *Nature Review Immunology*. **4**, 278-289.
10. van Ewijk W, Hollander G, Terhorst C, Wang B (2000). Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. *Development (Cambridge, England)*. 127, 1583-1591.
11. Andrea C Carpenter, Rémy Bosselut (2010). Decision checkpoints in the thymus. *Nature Review Immunology*. 11,666-673.

12. Ute Koch, Freddy Radtke (2011). Mechanisms of T cell Development and Transformation. *Annual Review. Cell Dev. Biol.* 092910-154008.
13. Yousuke Takahama (2006). Journey through the thymus: stromal guides for T-cell development and selection. *Nature Review Immunology*. 127-135.
14. Torroba M, Zapata AG (2003). Aging of the vertebrate immune system. *Microsc Res Tech*, 62: 477-481.
15. Thomas B.L. Kirkwood (2005). Time of our lives *EMBO reports*. 7400419.
16. AL Gruver, LL Hudson, GD Sempowski (2007). Immunosenescence of ageing. *Journal Pathology*.144-156.
17. Kirkwood TB (1977). Evolution of ageing. *Nature,* 270: 301-304.
18. Daryl P. *et al.* (2009). An evolutionary perspective on the mechanisms of immunosenescence. *Cell review*.
19. Hale, L.P. (2004). Histological and molecular assessment of human thymus. *Ann. Diagn. Pathol.* 8,50-60.
20. Min, B. *et al*. (2003) Neonates support lymphopenia-induced proliferation. *Immunity* 18, 131–140.
21. Howard JK *et al*. (1999). Leptin protects mice from starvation-induced lymphoid atrophy and increases thymic cellularity in ob/ob mice. *J Clin Inves*. 104:1051–1059.
22. Boehm T, Swann JB (2013). Thymus involution and regeneration: two sides of the same coin*? Nature reviews. Immunology*. 13, 831-838.
23. Tyan ML (1977). Age-related decrease in mouse T cell progenitors. *J Immunol*, 118:846-851.
24. Sieburg HB, *et al*.(2006). The hematopoietic stem compartment consists of a limited number of discrete stem cell subsets. *Blood.*;107(6):2311–6.

25. Kalpesh Patel et al (2009). Role of neuropeptides, hormones, and growth factors in regulating thymopoiesis in middle to old age. *Biology Reports* F1000 1:42.

26. Mackall CL, *et al*. (1998). Thymic function in young/old chimeras: substantial thymic T cell regenerative capacity despite irreversible age-associated thymic involution. *Eur J Immunol*, 28: 1886-1893.

27. Sutherland JS, *et al*. (2005). Activation of thymic regeneration in mice and humans following androgen blockade. *J Immunol.* 175(4):2741-53.

28. Olsen NJ, Olson G, Viselli SM, Gu X, Kovacs WJ. (2001). Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. *Endocrinology*.142(3):1278-83.

29. Guevara Patino JA, Marino MW, Ivanov VN, Nikolich-Zugich J. (2000). Sex steroids induce apoptosis of CD8+CD4+ double-positive thymocytes via TNF-alpha. *Eur J Immunol* ; 30: 2586–2592.

30. Zoller AL, Kersh GJ. (2006). Estrogen induces thymic atrophy by eliminating early thymic progenitors and inhibiting proliferation of beta-selected thymocytes. *J Immunol*; 176: 7371–7378.

31. Goldberg GL, *et al*. (2009). Luteinizing hormone-releasing hormone enhances T cell recovery following allogeneic bone marrow transplantation. *J Immunol* 182: 5846–5854.

32. E Velardi , J A Dudakov and M R M van den Brink (2015). Sex steroid ablation: an immunoregenerative strategy for immunocompromised patients. *Bone Marrow Transplantation* 50**,** S77–S81.

33. Lai KP, *et al*. (2013). Targeting thymic epithelia AR enhances T-cell reconstitution and bone marrow transplant grafting efficacy. *Mol Endocrinol* ; 27: 25–37.

34. Sutherland JS, *et al*. (2008). Enhanced immune system regeneration in humans following allogeneic or autologous hemopoietic stem cell transplantation by temporary sex steroid blockade. *Clin Cancer Res*; 14: 1138–1149.

35. Alpdogan O, *et al*. (2006). Keratinocyte growth factor (KGF) is required for postnatal thymic regeneration. *Blood.* ;107(6):2453-60.

36. Min D, *et al*. (2002). Protection from thymic epithelial cell injury by keratinocyte growth factor: a new approach to improve thymic and peripheral T-cell reconstitution after bone marrow transplantation. : *Blood.*;99(12):4592-600.

37. Beata Berent-Maoz, *et al*. (2011). Fibroblast growth factor-7 partially reverses murine thymocyte progenitor aging by repression of *Ink4a*. *Blood.*; 119(24): 5715–5721.

38. Cesar O. *et al*. (2004). Phase I/II Randomized Trial Evaluating the Safety and Clinical Effects of Repifermin Administered to Reduce Mucositis in Patients Undergoing Autologous Hematopoietic Stem Cell Transplantation. *Clin Cancer Res* 1078-0432.

39. J Filicko, H M Lazarus and N Flomenberg. (2003). Mucosal injury in patients undergoing hematopoietic progenitor cell transplantation: new approaches to prophylaxis and treatment. *Bone Marrow Transplantation* 10.1038/sj.bmt.1703776.

40. Dixit VD, *et al*. (2007). Ghrelin promotes thymopoiesis during aging. *J Clin Invest.* 2778-90.

41. A Inui, *et al*. (2004). Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ , *The FASEB Journal*, 18(3):439-56.

42. Crystal L. Mackall, Terry J. Fry and Ronald E. Gress. (2011). Harnessing the biology of IL-7 for therapeutic application. *Nature Reviews Immunology* 11, 330-342.

43. Lu H, *et al*. (2005). Interleukin-7 improves reconstitution of antiviral CD4 T cells. *Clin Immunol.* 114(1):30-41.

44. Sportes, C. *et al.* (2008).Administration of rhIL-7 in humans increases *in vivo* TCR repertoire diversity by preferential expansion of naive T cell subsets. *J. Exp. Med.* 205, 1701–1714.

45. Rosenberg, S. A. *et al.* (2006). IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells. *J. Immunother.* 29, 313–319

46. Jarrod A. Dudakov, Alan M. Hanash and Marcel R.M. van den Brink. (2015). Interleukin-22: Immunobiology and Pathology. *Annual Review of Immunology*.33: 747-785.

47. Pan B, *et al*. (2014). Acute ablation of DP thymocytes induces up-regulation of IL-22 and Foxn1 in TECs. *Clin Immunol.* 150(1):101-8.

48. Dudakov JA, *et al*. (2012). Interleukin-22 drives endogenous thymic regeneration in mice. *Science*. 6;336(6077):91-5.

49. Rosanne Spolski & Warren J. Leonard (2014). Interleukin-21: a double-edged sword with therapeutic potential. *Nature Reviews Drug Discovery* 13(5):379-95.

50. Sugamura K, *et al*. (1996). The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Annu Rev Immunol.* 1996;14:179-205.

51. Zhou L, *et al*. (2007). IL-6 programs $T_H$-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* 8:967–74.

52. Megan A. Luckey (2013). γc cytokine signaling: graduating school in thymic education. *Blood* 121.1.

53. Li Y, Bleakley M, Yee C. (2005). IL-21 influences the frequency, phenotype, and affinity of the antigen-specific CD8 T cell response. *J. Immunol.* 175:2261–69.

54. Jarrod A. Dudakov *et al* (2010). Feeding the fire: the role of defective bone marrow function in exacerbating thymic involution. *Trends in Immunology* 31(5) 191-198.

55. Ozaki K, *et al*. (2002). A critical role for IL-21 in regulating immunoglobulin production. *Science* 298:1630–34.

56. Rafei M, Dumont-Lagace M, Rouette A, Perreault C (2013). Interleukin-21 accelerates thymic recovery from glucocorticoid-induced atrophy. *PloS one*. 8, e72801.

57. Rafei M, Rouette A, Brochu S, Vanegas JR, Perreault C (2013). Differential effects of gammac cytokines on postselection differentiation of CD8 thymocytes. *Blood*. 121, 107-117.

58. Dudakov JA, Khong DM, Boyd RL, Chidgey AP (2010). Feeding the fire: the role of defective bone marrow function in exacerbating thymic involution. *Trends Immunol*. 31(5):191-8.

59. Mehmood H Hashmi & Peter J Van Veldhuizen, (2010). Interleukin-21: updated review of phase I and II clinical trials in metastatic renal carcinoma, metastatic melanoma and relapsed/refractory indolent non-Hodgkin's lymphoma. *Expert Opinion on Biological Therapy*. 10(5) 807-817.

60. Frederiksen KS, Lundsgaard D, Freeman JA, *et al* (2012). IL-21 induces in vivo immune activation of NK cells and CD8(+) T cells in patients with metastatic melanoma and renal cell carcinoma.Aging *Cell*; 11(5):732-40.

61. Honh He *et al* (2006). Combined IL-21 and Low-Dose IL-2 therapy induces anti-tumor immunity and long-term curative effects in a murine melanoma tumor model. *J Transl Med*. 4:24.

*Aging Cell* (2016) **15**, pp349–360 Doi: 10.1111/acel.12440

# Interleukin-21 administration to aged mice rejuvenates their peripheral T-cell pool by triggering *de novo* thymopoiesis

E. Al-Chami,[1] A. Tormo,[1] S. Pasquin,[1] R. Kanjarawi,[1] S. Ziouani[2] and M. Rafei[1]

[1]Department of Pharmacology, Université de Montréal, Montréal, QC H3C 1J7, Canada
[2]Université Paris-Sud, Faculté de Pharmacie, 5 rue J.B. Clément, 92296 Châtenay-Malabry Cedex, France

## Summary

**The vaccination efficacy in the elderly is significantly reduced compared to younger populations due to thymic involution and age-related intrinsic changes affecting their naïve T-cell compartment. Interleukin (IL)-21 was recently shown to display thymostimulatory properties. Therefore, we hypothesized that its administration to ageing hosts may improve T-cell output and thus restore a competent peripheral T-cell compartment. Indeed, an increase in the production of recent thymic emigrants (RTEs) attributable to intrathymic expansion of early thymic progenitors (ETPs), double-negative (DN), and double-positive (DP) thymocytes as well as thymic epithelial cell (TEC) was observed in recombinant (r)IL-21-treated aged mice. In sharp contrast, no alterations in the frequency of bone marrow (BM)-derived progenitors were detected following rIL-21 administration. Enhanced production of naïve T cells improved the T-cell receptor (TCR) repertoire diversity and re-established a pool of T cells exhibiting higher levels of miR-181a and diminished amounts of the TCR-inhibiting phosphatases SHP-2 and DUSP5/6. As a result, stimulation of T cells derived from rIL-21-treated aged mice displayed enhanced activation of Lck, ZAP-70, and ERK, which ultimately boosted their IL-2 production, CD25 expression, and proliferation capabilities in comparison with T cells derived from control aged mice. Consequently, aged rIL-21-treated mice vaccinated using a tyrosinase-related protein 2 (Trp2)-derived peptide exhibited a substantial delay in B16 tumor growth and improved survival. The results of this study highlight the immunorestorative function of rIL-21 paving its use as a strategy for the re-establishment of effective immunity in the elderly.**

**Key words: interleukin-21; miR-181a; T cells; thymic involution; tumor vaccine; signaling.**

## Introduction

In addition for being the key site of T lymphopoiesis in jawed vertebrates, the thymus maintains a competent peripheral T-cell pool with a broad spectrum of TCR specificities (Lynch *et al.*, 2009). It is, however, well established that immunity declines with ageing owing to two key factors impeding thymic function: a defect in the survival/proliferation ability of the prethymic hematopoietic progenitor pool coupled to the precocious loss of TECs (Boehm & Swann, 2013). These age-related changes, collectively known as thymic involution, represent major driving forces for homeostatic expansion of preexisting peripheral T cells (Lynch *et al.*, 2009). The net outcome culminates in TCR repertoire skewing with a noticeable increase in the number of effector/memory T cells (Zanni *et al.*, 2003). Notably, a growing body of literature established that while the size of the peripheral T-cell compartment remains unchanged throughout ageing, an increase in post-thymic lifespan of T cells takes place consequently leading to the emergence of T-cell intrinsic defects (Haynes & Swain, 2006; Maue *et al.*, 2009; Tsukamoto *et al.*, 2009). For instance, naïve $CD4^+$ T cells derived from aged mice display defects in TCR threshold calibration, do not readily form immunological synapses, and have a marked reduction in the recruitment of TCR-associated signaling molecules when compared to younger mice (Garcia & Miller, 1997, 2001, 2002; Tamir *et al.*, 2000). Furthermore, increased expression of inhibitory receptors such as PD1, LAG3, 2B4, and CD160 were observed on the surface of ageing $CD8^+$ T cells (Decman *et al.*, 2012), while both IL-2 secretion and proliferation potential are limited in naïve $CD4^+$ and $CD8^+$ T cells derived from aged mice (Eaton *et al.*, 2004). Thus, stifled thymopoiesis combined to global qualitative changes affecting the ageing peripheral T-cell pool limits the host's ability to mount effective responses against new antigenic challenges and accounts for the eroded immunity commonly observed in the elderly.

Primarily produced by activated $CD4^+$ T cells, IL-21 is a prominent member of the common γ-chain family of cytokines (Spolski & Leonard, 2014). Besides its wide-ranging effects on immune cells, IL-21 overexpression *in vivo* triggers expansion of BM-derived progenitors (Ozaki *et al.*, 2006). Furthermore, we recently reported a novel mitogenic function for IL-21 on peptide-mediated TCR-engaged DP thymocytes using a newly developed *in vitro* coculture system designed for T-cell differentiation (Rafei *et al.*, 2013b). Likewise, rIL-21 administration to mice with glucocorticoid-induced thymic atrophy dramatically accelerates thymic function recovery by stimulating the proliferation of ETPs, DN, and positively selected DP thymocytes (Rafei *et al.*, 2013a). Such unprecedented thymopoiesis-supporting function suggests that rIL-21 is indeed a promising therapeutic tool endowed with the capacity of improving T-cell output in aged hosts owing to the expression of the IL-21 receptor (IL-21R) on both BM and thymic progenitors (Ozaki *et al.*, 2006; Rafei *et al.*, 2013a,b). We wished therefore to scrutinize whether rIL-21 administration to aged mice can rejuvenate their T-cell immunity by targeting *de novo* thymopoiesis as a mean to enhance their antitumoral response following vaccination.

## Results

### Administration of rIL-21 enhances thymopoiesis in aged mice

To ensure maximal thymopoiesis-stimulating effects *in vivo*, we first conducted a dose–response study in young (2 months—2M) versus old (15 months—15M) RAG2p-GFP mice by intraperitoneally (IP) administering rIL-21 (Fig. S1A). The use of the RAG2p-GFP model allows to easily

Correspondence
*Moutih Rafei, PhD, Department of Pharmacology, Université de Montréal, PO Box 6128, Downtown Station, Montréal, QC H3C 3J7, Canada. Tel.: (514) 343-6931; fax: (514) 343-2291; e-mail:* [redacted]

Accepted for publication *29 November 2015*





Aging Cell

assess *de novo* thymopoiesis as expression of the GFP transgene, marking newly developed T cells, is controlled by the *Rag2* promoter activity (Monroe *et al.*, 1999; Yu *et al.*, 1999; Rafei *et al.*, 2011). Thymic analysis 1 week following the last injection revealed a progressive increase in total thymocyte count in 15M but not 2M-old rIL-21-treated mice with an optimal response rate achieved at a dose of 50 μg $kg^{-1}$ (Fig. S1B). Similarly, only aged mice receiving rIL-21 exhibited an increase in the counts of all thymic subsets (DN, DP and single positive) (Fig. 1A) including ETPs (Fig. 1B,C). Even though the percentage of $GFP^+$ thymocytes remained unchanged in all studied groups (Fig. S1C), the increased thymic count observed in the rIL-21-treated aged mice was sustained over a 3-week period postcytokine administration (Fig. S1D). To determine whether rIL-21-enhanced thymopoiesis involves the expansion of BM progenitors, which could have increased their migration rate to the thymus, we next monitored the frequency of LSK cells ($Lin^-$ $Sca1^+$ $c\text{-}Kit^+$) and its subpopulations including the long-term (LT; $Lin^-$ $Sca1^+$ $c\text{-}Kit^+$ $CD34^-$ $CD135^-$) and short-term (ST; $Lin^-$ $Sca1^+$ $c\text{-}Kit^+$ $CD34^+$ $CD135^-$) hematopoietic stem cells (HSCs), as well as multipotent progenitors (MPPs; $Lin^-$ $Sca1^+$ $c\text{-}Kit^+$ $CD34^+$ $CD135^+$) following rIL-21 treatment. Despite IL-21R expression on the surface of wild-type (WT) LT, ST-HSCs, and MPPs (Fig. S2A), the overall proportion of LSK cells (Fig. S2B) or its subpopulations (Fig. S2C) was not affected by rIL-21 administration. Likewise, no increase in the number of LSK subpopulations (Fig. S2D) nor in the more differentiated common lymphoid progenitor (CLP; $Lin^-$ $IL\text{-}7R^+$ $Sca1^+$ $c\text{-}Kit^+$) population (Fig. S2E) was observed. Furthermore, only *in vitro* cultured young LSK cells proliferated when cultured with rIL-21 (Fig. S2F) clearly suggesting a defective response in ageing BM.

We recently reported that TECs are devoid of IL-21R (Rafei *et al.*, 2013a). Therefore, we presumed that the thymic effects observed following rIL-21 infusion strictly affect hematopoietic cells. Indeed, when given to aged mice, rIL-21 does not fluctuate the frequency of total TECs ($EpCAM^+$ $CD45^-$), nor it did affect the ratio of cortical (c)TEC ($EpCAM^+$ $CD45^-$ $UAE\text{-}1^-$ $Ly51^+$) relative to medullary (m)TEC ($EpCAM^+$ $CD45^-$ $UAE\text{-}1^+$ $Ly51^{-/lo}$) populations (Fig. 1D,E). Conversely, absolute counts analysis showed marked improvements in the stromal compartment as total, cTEC, and mTEC populations were significantly higher in rIL-21-treated aged mice compared to the PBS group (Fig. 1F). Higher production of IL-7/thymus production was also noticed in the rIL-21-treated aged mice (Fig. S1E). These data suggest that rIL-21 administration is beneficial to aged mice by directly targeting thymopoiesis *in situ* without triggering the expansion of BM-derived LSK cells.

### Physiological levels of RTE are restored in aged mice following rIL-21 treatment

To interrogate the functional relevance of rIL-21-enhanced thymopoiesis on the peripheral T-cell pool of aged RAG2p-GFP mice, we next assessed the percentage of circulating RTEs mirrored by the level of peripheral $GFP^+$ $CD3\varepsilon^+$ T cells. In contrast to 2M-old animals, where RTEs represent roughly 2.3% of total circulating lymphocytes, lower percentages (~0.5%) are detected in the peripheral blood of 15M PBS-treated aged mice (Fig. 2A). Following rIL-21 treatment, the percentage of $GFP^+$ $CD3\varepsilon^+$ T cells reached a range of 1.3–1.7% over a period of 3-week postcytokine treatment (Fig. 2A,B) with absolute numbers attaining physiological levels according to RTE counts calculated using blood derived from young mice (Fig. 2C).

With increased encountered antigens and declined RTE levels, qualitative changes in the phenotype of peripheral T-cell composition occur with ageing (Boursalian *et al.*, 2004). More specifically, the expression of various cell surface makers including the glycoprotein CD45RB is downregulated as T cells become activated and progress from a naïve to a memory phenotype (Tough & Sprent, 1994). We therefore hypothesized that enhancing RTE generation in aged mice would increase the overall expression pattern of CD45RB on peripheral T-cell pool and found that it was indeed the case in aged mice treated with rIL-21 as depicted by histogram overlaps (Fig. 2D) and compiled mean fluorescent intensities (MFIs) (Fig. 2E). We therefore conclude that rIL-21 treatment enhances the *de novo* generation of RTEs, which incorporate the peripheral T-cell pool of aged mice.

### The nature of ageing T-cell pool is greatly affected by rIL-21 administration

Following thymic egress, RTEs continue their maturation in the periphery to eventually become fully competent mature naïve T cells (Boursalian *et al.*, 2004). To do so, they require access to secondary lymphoid organs (SLO) as a mean to encounter other cell types and cytokines required for their maturation (Houston *et al.*, 2008). Although the percentage of $GFP^+$ T cells increased significantly in the spleen of rIL-21-treated aged mice in the 3 weeks following cytokine treatment (Fig. 3A), the overall number of splenocytes remained steady (Fig. 3B). Further in-depth analysis revealed a progressive time-dependent increase in the absolute counts of $GFP^+$ $CD4^+$ and $GFP^+$ $CD8^+$ T cells in the spleens of rIL-21-treated aged mice (Fig. 3C) suggesting that SLO-resident aged T cells were displaced by newly migrating RTEs. We next examined by flow cytometry the differentiation stages of spleen-derived $CD4^+$ and $CD8^+$ T cells and detected increased abundance of T cells with a naïve phenotype ($CD44^{lo}CD62L^{hi}$) in IL-21-treated aged mice (Fig. 3D,E) bolstering the notion of rIL-21-mediated enhanced T-cell output.

Given that thymic involution compromises the TCR repertoire (Yager *et al.*, 2008; Ahmed *et al.*, 2009), we continued our analysis by investigating the effect of rIL-21 administration on TCR diversity. To address this question, the expression profile of 15 TCRVβ-chains was analyzed on the surface of spleen-derived $CD4^+$ and $CD8^+$ T cells collected from treated mice. Although no changes occurred in the TCRVβ-chains of young mice treated with PBS or rIL-21 (Fig. S3), significant improvements were observed in the proportion of Vβ2-, 6-, 8.1/8.2-, 11-, 12-, and 14-expressing $CD4^+$ T cells as well as Vβ2-, 5.1/5.2-, 6-, 8.1/8.2-, 8.3-, 11-, and 12-expressing $CD8^+$ T cells following rIL-21 administration to ageing mice (Fig. 3F). These results indicate that rIL-21-mediated *de novo* RTE generation is associated with major qualitative changes in the peripheral T-cell pool of aged mice including improved TCR diversity.

### Characterizing the biochemical responses of T cells

Thymic involution cannot solely account for impaired immune responses as additional T-cell intrinsic defects appear in ageing naïve T cells due to prolonged post-thymic lifespan (Haynes & Swain, 2006; Maue *et al.*, 2009; Tsukamoto *et al.*, 2009). More specifically, TCR activation is blunted with ageing due to increased cytoplasmic concentration of phosphatases known for inhibiting TCR signaling (Li *et al.*, 2012). Given such fluctuation in TCR threshold calibration, we next explored whether the previously observed changes in the nature of the peripheral T-cell pool induced by rIL-21 affect the expression levels of the TCR-targeting phosphatases SHP-2, PTPN-22, and DUSP5/6. Delineation of phosphatase levels by Western blotting (Fig. 4A) and densitometry-based quantification (Fig. 4B) revealed diminished SHP-2 and DUSP5/6 levels in

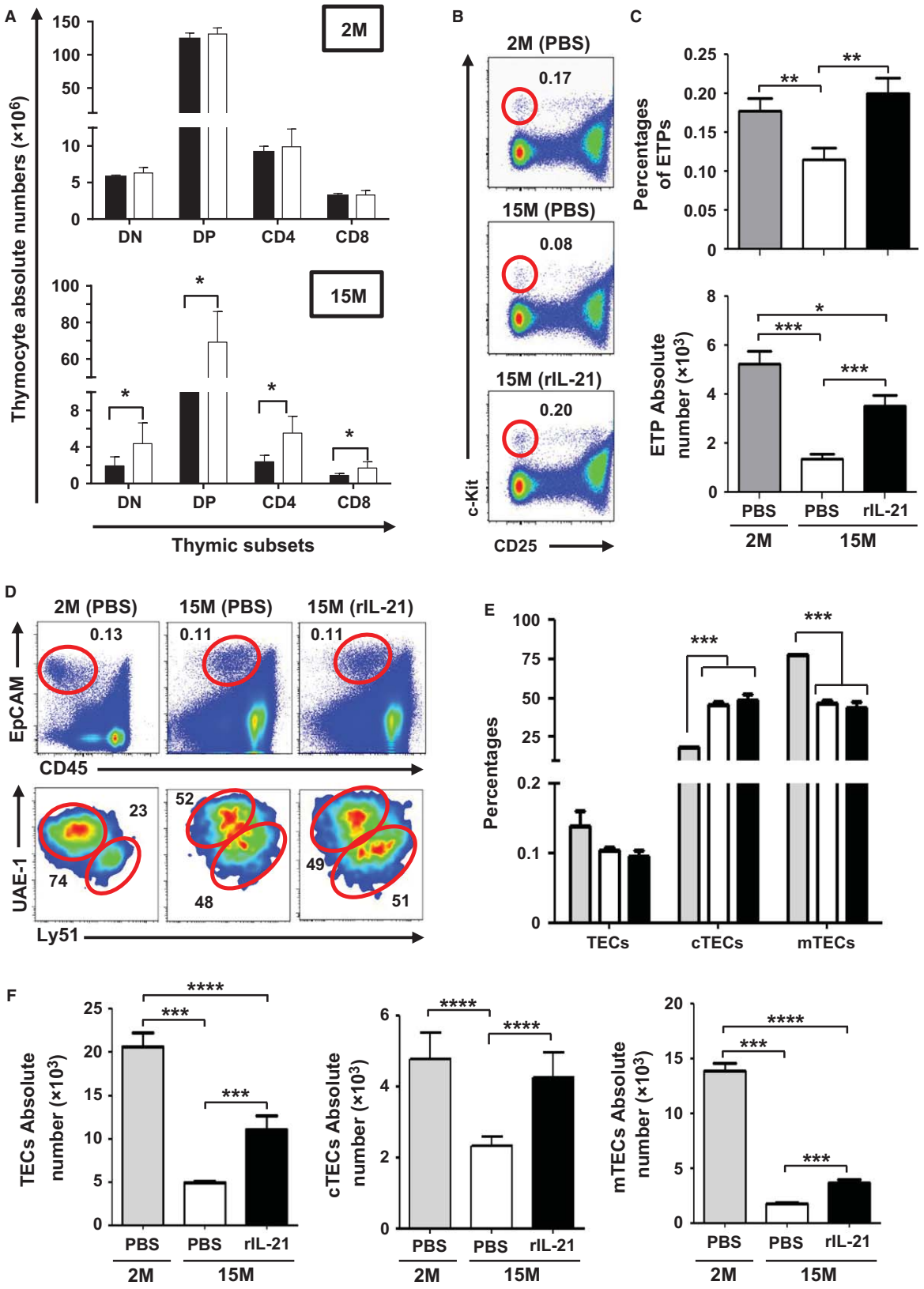



**Fig. 1** Administration of rIL-21 promotes *de novo* thymopoiesis in aged but not young mice. (A) Counts of thymocyte subpopulations. Groups are displayed as PBS (■) and IL-21 (□). (B) Representative flow cytometry analysis of ETPs. (C) Absolute count of ETPs. (D, E) Percentages of total, cTECs, and mTECs. (F) Absolute counts of total, cTECs, and mTECs in comparison with PBS-treated aged mice. For panels C, E, and F, groups are displayed as: 2M (PBS ▫), 15M (PBS □), or 15M (rIL-21 ■). All data are representative of three independent experiments ($n$ = 5/group with $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$).

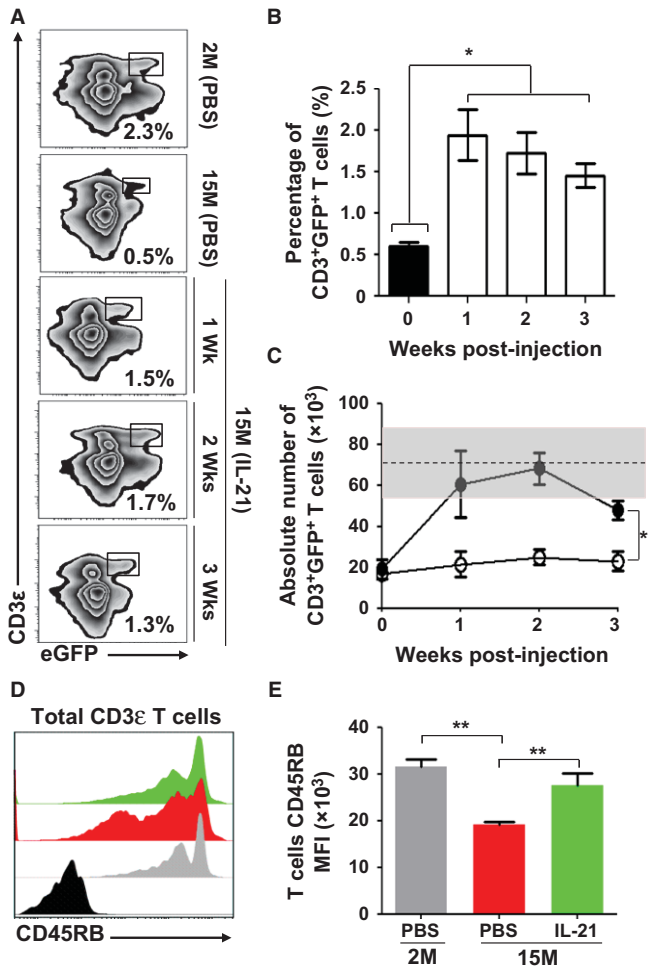



**Fig. 2** Increased levels of circulating RTEs in rIL-21-treated aged mice. (A) Representative flow cytometry analysis of RTEs in peripheral blood of young (2M) or aged (15M) mice 1, 2, or 3 weeks post-rIL-21 administration. Young mice treated with PBS were used as comparative positive controls. (B, C) Analysis of overall percentages (B) and counts (C) of RTEs in the weeks following rIL-21 administration to aged mice. The black histogram represents the 2M PBS-treated mice. The gray zone in (C) represents the RTE level calculated using 2M young mice ($n$ = 10) and displayed as the average RTE number ± two SD. Black circles in (C) represent rIL-21-treated mice. (D) Representative flow cytometry analysis of CD45RB on the surface of all $CD3^+$ T cells derived from 2M (PBS□), 15M (PBS■), and 15M (rIL-21■). CD45RB isotype is displayed in black. (E) Compiled MFIs for CD45RB expression in treated mice. All data are representative of three independent experiments ($n$ = 5/group with $^*P < 0.05$ and $^{**}P < 0.01$).

T cells derived from rIL-21-treated aged mice at all tested time points. Only PTPN-22 remained unchanged in all groups throughout treatments (Fig. 4A,B). Previous studies conducted in mice demonstrated that SHP-2 and DUSP5/6 were among several phosphatases controlled by miR-181a, a ~22nt microRNA molecule capable of repressing the translation of over 40 phosphatases (Li *et al.*, 2007). Consistently, the progressive decline in miR-181a levels observed in ageing human naïve $CD4^+$ T cells dovetails the decreased T-cell responsiveness following TCR stimulation (Li *et al.*, 2012). To examine the possibility that rIL-21 could have reversed such defect through enhanced generation of RTEs expressing normal levels of miR-181a, quantitative (q)PCR studies were conducted using T cells sorted from spleens of treated mice. Our analysis revealed a two- to threefold increase in miR-181a levels in T cells derived from rIL-21-treated aged mice (Fig. 4C), which is consistent with the diminished SHP-2 and DUSP5/6 levels observed earlier (Fig. 4A,B). We next tested the responses of T cells derived from treated mice by probing early signaling events triggered by TCR stimulation. Although not efficient as younger lymphocytes, $CD4^+$ or $CD8^+$ T cells derived from rIL-21-treated aged mice displayed higher Lck, ZAP-70, and ERK phosphorylation compared to control PBS aged mice as shown by phospho-flow analysis (Fig. 4D). Taken collectively, these data indicate that enhanced TCR responses in the pool of T cells derived from rIL-21-treated aged mice are attributable to the increase in the proportion of naïve T cells displaying lower SHP-2 and DUSP5/6 phosphatase levels owing to improved miR-181a expression.

## rIL-21 administration improves the biological responses of T cells derived from aged mice

The dramatic reduction in naïve T-cell output coupled to the relative increase in the proportion of effector and central memory T cells in aged mice negatively impact T-cell responses to neo-antigens (Yager *et al.*, 2008). Repeated observations revealed that beside scarcity of T-cell precursors in the pre-immune repertoire, IL-2 production, cell surface expression of CD25, and T-cell proliferation are all greatly reduced in ageing hosts (Haynes & Swain, 2006; Maue *et al.*, 2009; Tsukamoto *et al.*, 2009). Elevated secretion levels of pro-inflammatory cytokines such as interferon (IFN)γ caused by the accumulation of $CD44^{hi}$ T cells were also reported to play a role in accelerating age-related immunosenescence (Zhang *et al.*, 2002; Decman *et al.*, 2012). The observations made so far led us to speculate that the beneficial effect of rIL-21 on thymopoiesis in aged mice should improve the effector function of their rejuvenated T-cell pool. To test this hypothesis, spleen-derived $CD3\varepsilon^+$ T cells were isolated 3 weeks following the last cytokine treatment (to ensure that they had completed their necessary post-thymic maturation) and stimulated using CD3-CD28 beads (Berkley *et al.*, 2013). Not only were IFNγ and IL-2 secretion profiles by TCR-stimulated T cells derived from rIL-21-treated mice comparable to younger animals, but a striking decrease in IL-17 production was observed as well (Fig. 5A). In addition, the intensity of CD25 cell surface expression on spleen-purified TCR-stimulated $CD4^+$ or $CD8^+$ T cells and their proliferation rates were comparable between young and rIL-21-treated aged mice as determined by flow cytometry (Fig. 5B,D) and compiled MFIs data (Fig. 5C,E). To gain further insights, we quantified by qPCR the expression level of several genes known to influence the differentiation and/or effector function of T cells. Although no difference was observed in *Nfat*, *Ap-1,* and *Gata3* expression in all tested groups, a decrease in *T-bet* and *RORC* (encoding for RORγt) transcript levels was detected in T cells derived from rIL-21-treated aged mice compared to aged PBS control mice (Fig. S4). *Foxp3* expression level, on the other hand, was equivalent between PBS- and rIL-21-treated aged mice but significantly higher than younger mice (Fig. S4). These qPCR results are consistent with the observed decrease in IFNγ (*T-bet*) and IL-17 (*RORC*) secretion levels and are particularly interesting as several investigations have associated IL-21 to autoimmune diseases (Korn *et al.*, 2007; Peluso *et al.*, 2007; Attridge *et al.*, 2012). Further analyses conducted on rIL-21-treated aged mice showed no unusual signs of inflammation or autoimmunity (Fig. S5). More specifically, we found (i) that the spleen size and weight of PBS- or rIL-21-treated aged mice were similar to younger mice (Fig. S5A), (ii) that the total serum IgG levels were lower in young mice but comparable in both PBS- and rIL-21-treated animals indicating an age-related factor at play independent of rIL-21 treatment (Fig. S5B), and (iii) no immune infiltrates in lungs, liver, or kidneys of aged mice receiving rIL-21 (Fig. S5C). In agreement with previous studies reporting accumulation of

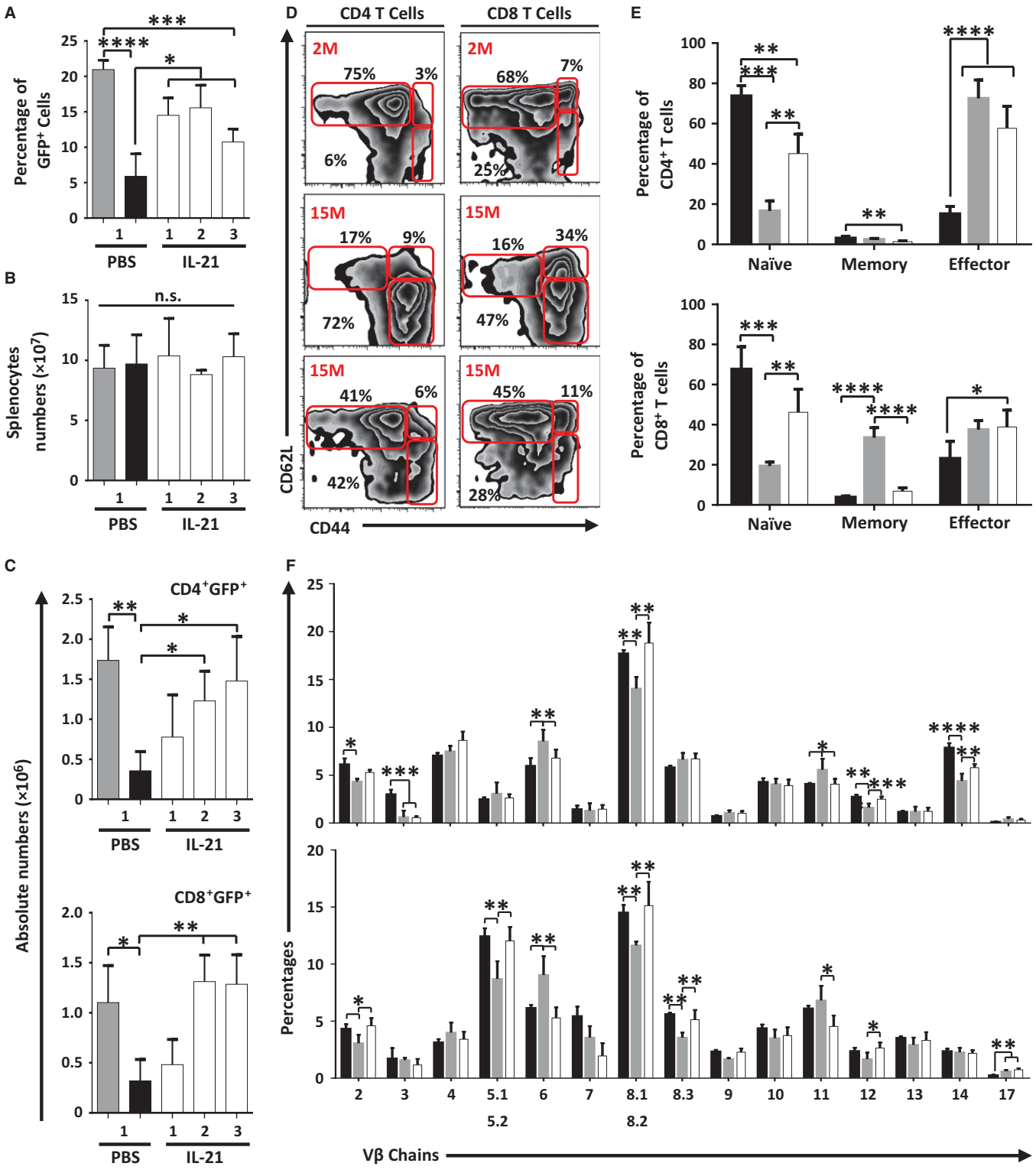



**Fig. 3** Aged mice treated with rIL-21 display increased proportion of naïve T cells with enhanced TCR diversity. (A) Percentages of GFP$^+$ events in the spleen of treated mice. (B) Splenocyte counts in all experimental groups. (C) Absolute counts of CD4$^+$ GFP$^+$ and CD8$^+$ GFP$^+$ T cells in treated mice. (D) A representative flow cytometry analysis of naïve (CD62L$^{hi}$CD44$^{lo}$), memory (CD62L$^{hi}$CD44$^{hi}$), and effector (CD62L$^{lo}$CD44$^{hi}$) T cells in all experimental groups. (E) Compiled percentages of all three subpopulations in CD4$^+$ (top panel) and CD8$^+$ (lower panel) T cells. (F) Flow cytometry analysis of 15 TCRVβ chains using peripheral CD4$^+$ (top panel) or CD8$^+$ (lower panel) T cells. For panels A–C, groups are displayed as 2M (PBS □), 15M (PBS ■), and 15M (rIL-21 □). For panels E and F, groups are displayed as 2M (PBS ■), 15M (PBS □), and 15M (rIL-21 □). All data are representative of three independent experiments ($n$ = 5/group with *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001, and ****$P$ < 0.0001).

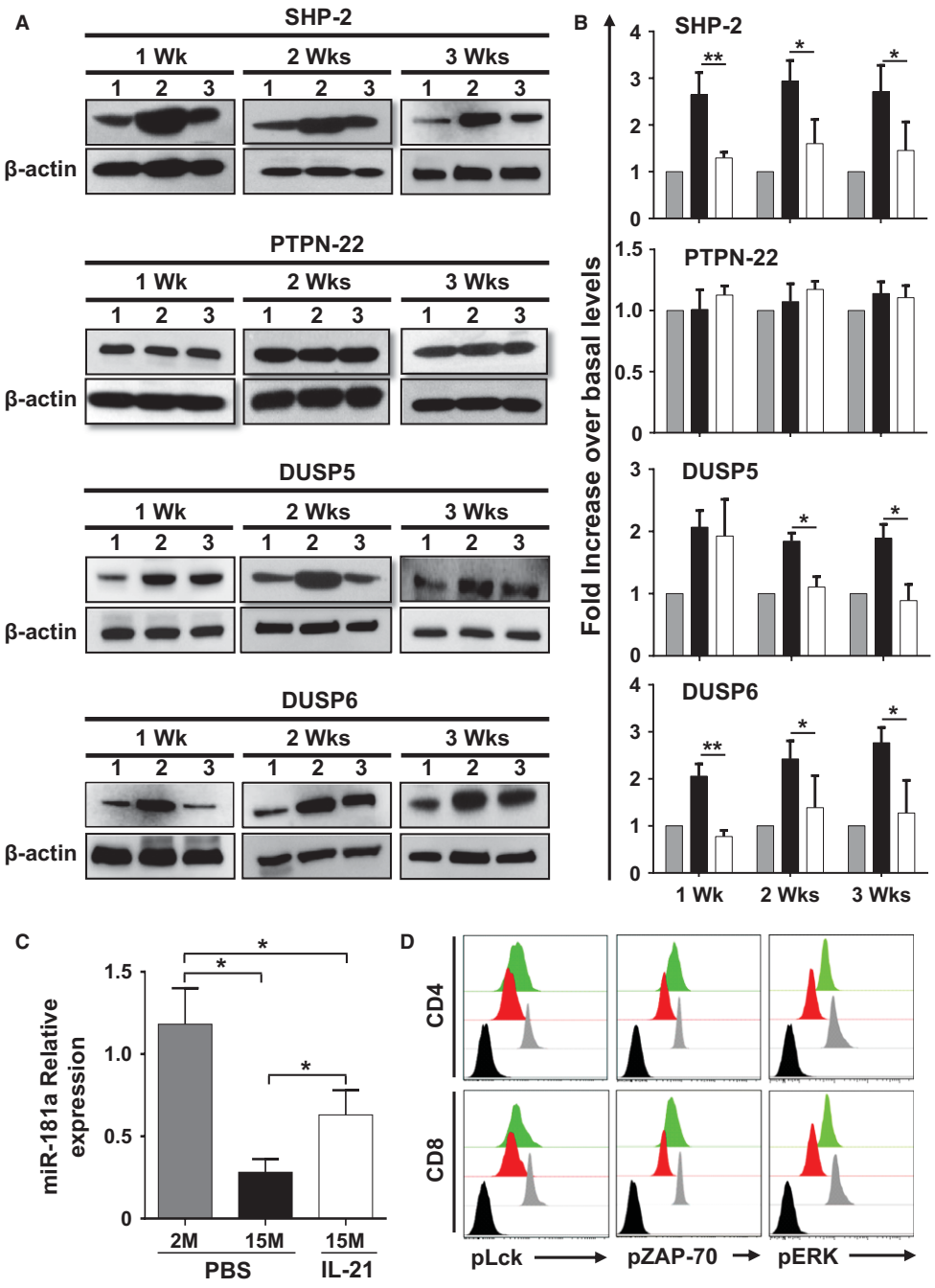



**Fig. 4** The peripheral pool of T cells in rIL-21-treated aged mice displays improved TCR signaling responses. (A) Representative Western blot analyses of phosphatases at 1, 2, or 3 weeks post-treatments of 2M PBS (1), 15M PBS (2), and 15M rIL-21 (3) aged mice. β-actin was used as internal loading control. (B) Compiled densitometry analysis of phosphatase expression levels. The displayed groups are as follows: 2M (PBS□), 15M (PBS ■), and 15M (rIL-21 □) aged mice. C) qPCR analysis of miR-181a in freshly isolated T cells from 2M (PBS □), 15M (PBS ■), and 15M (rIL-21 □) treated mice. D) Representative intracellular flow cytometry staining of pLck, pZAP-70, and pERK in 2M (PBS □), 15M (PBS ■), and 15M (rIL-21 ■) aged mice. Nonstimulated T cells derived from young 2M mice are displayed by black histograms. All data are representative of three independent experiments ($n$ = 5/group with $^{*}P < 0.05$, and $^{**}P < 0.01$).

$CD25^{+}$ $CD4^{+}$ $T_{regs}$ in the periphery and SLO of ageing mice and humans (Lages *et al.*, 2008), we detected a significantly higher percentage and absolute number of spleen-derived $T_{regs}$ in both PBS- and rIL-21-treated aged mice when compared to their younger counterparts (Fig. S5D,E). Globally, these findings indicate that rIL-21-mediated boosting of T-cell output in aged mice corrects the T-cell dysfunctionalities commonly seen with ageing without inducing signs of autoimmunity.

### Enhanced vaccine-elicited antitumoral response in rIL-21-treated aged mice

To evaluate the effectiveness of our T-cell rejuvenation therapy, a proof-of-concept vaccination study was conducted using an experimental melanoma antigen (Parkhurst *et al.*, 1998). Briefly, aged mice were first treated with PBS or rIL-21 according to our established

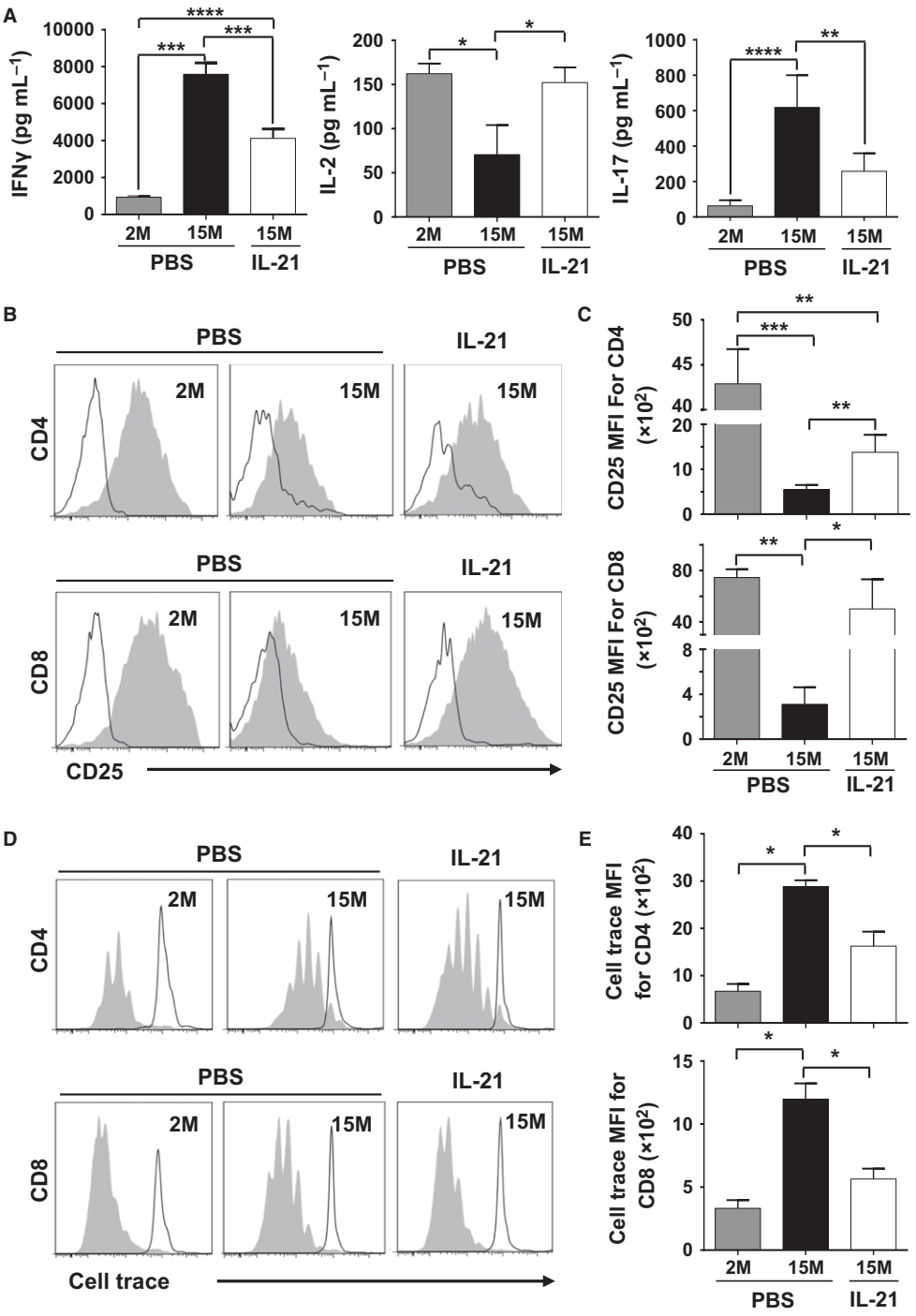



**Fig. 5** Enhanced biological responses of peripheral T-cell pool derived from rIL-21-treated aged mice. (A) Cytokine quantification from stimulated CD3$^+$ T cells isolated from the spleen of treated mice. (B) Representative flow cytometry analysis of CD25 cell surface expression on CD4$^+$ (top panels) and CD8$^+$ (lower panels) T cells. (C) Compiled MFIs for CD25 expression on CD4$^+$ and CD8$^+$ T cells. (D) Representative flow cytometry analysis of cell trace dilution following TCR stimulation of CD4$^+$ (top panels) and CD8$^+$ (lower panels) T cells derived from treated animals. (E) Compiled MFIs for cell trace dilution of CD4$^+$ and CD8$^+$ T cells following stimulation. All data are representative of three independent experiments ($n$ = 5/group with *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001, and ****$P$ < 0.0001).

protocol, then left for 3 weeks to allow for RTE maturation (Fig. 6A). All mice were then vaccinated weekly (for a total of three injections) using *in vitro* generated BM-derived mature DCs (Fig. S6) pulsed with SIINFEKL (a control epitope derived from chicken ovalbumin) or SVYDFFVWL peptides (the experimental epitope derived from the melanoma differentiation antigen Trp2). Two weeks following the last DC boost, mice were challenged subcutaneously (SC) with syngeneic B16 tumor cells and monitored thereafter for tumor growth and survival. Although all SIINFEKL-vaccinated mice died within the same time frame (day 20–24) with no statistically significant difference

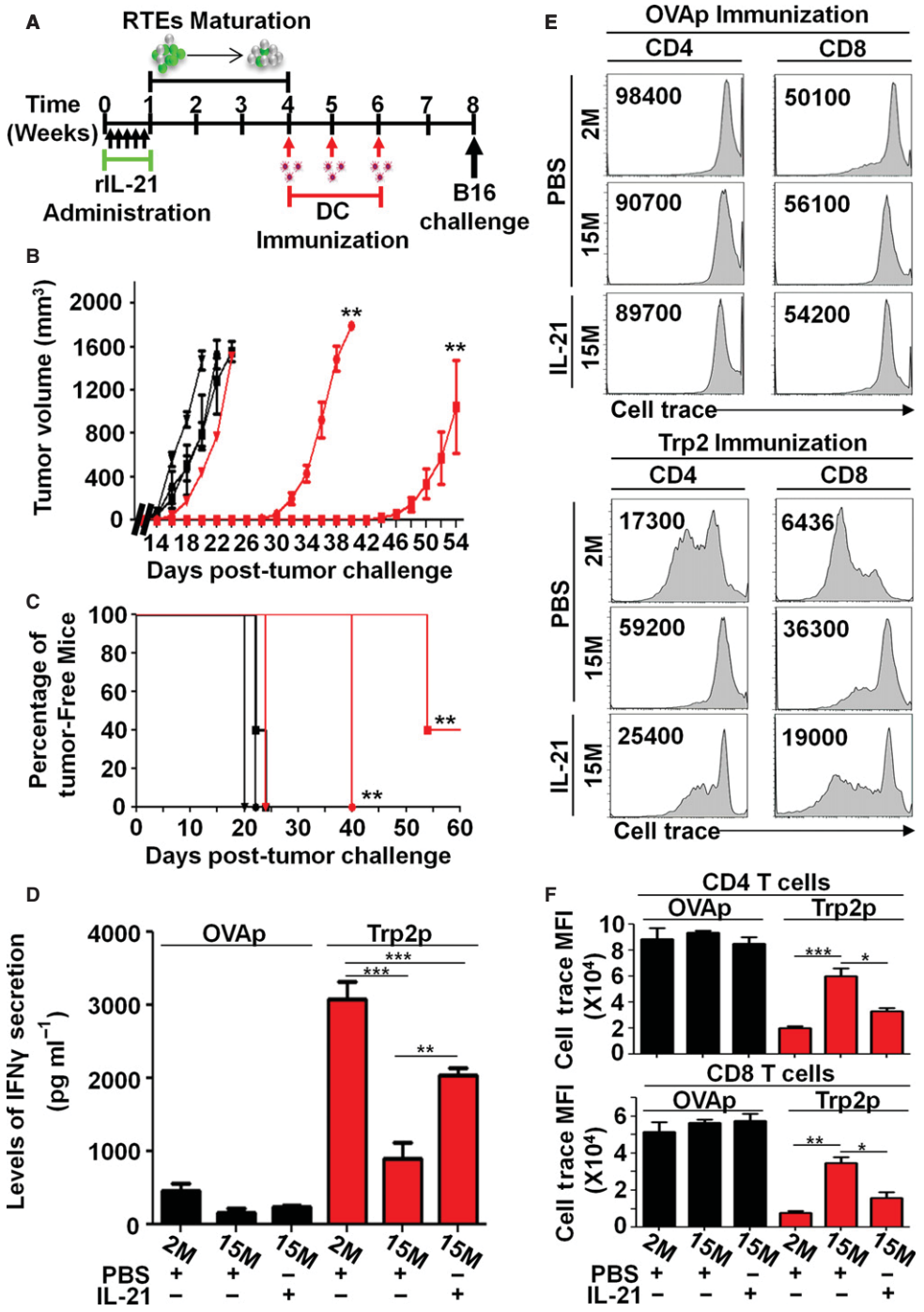



**Fig. 6** T-cell rejuvenation of aged mice enhances their antitumoral immunity. (A) Timeline used for vaccination. (B, C) Tumor volumes and percentage of tumor-free mice post-B16 tumor cell challenge in OVAp-DC-vaccinated 2M (PBS ■), 15M (PBS ▼), and 15M (rIL-21 ●); or Trp2p-DC-vaccinated 2M (PBS ■), 15M (PBS ▼) and 15M (rIL-21 ●). (D) IFNγ quantification following *in vitro* Trp2p stimulation of T cells derived from vaccinated animals. (E) Representative flow cytometry analysis of cell trace dilution in $CD4^+$ and $CD8^+$ T cells derived from vaccinated animals following *in vitro* Trp2 stimulation. (F) Compiled MFIs for cell trace dilutions in $CD4^+$ (top panel) and $CD8^+$ (lower panel) T cells following *in vitro* Trp2 stimulation. OVAp (SIINFEKL) and Trp2p (SVYDFFVWL). All data are representative of three independent experiments ($n$ = 5/group with $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

between young, PBS-, or rIL-21-treated mice, a slower tumor growth (Fig. 6B) and a significant delay in survival (Fig. 6C) were observed in the Trp2-vaccinated rIL-21-treated aged mice in comparison with the Trp2-vaccinated PBS control group (survived up to 40 versus 22 days, respectively). We then retrieved the spleens of mice from each group and analyzed their IFNγ production level and proliferation following Trp2 restimulation *in vitro*. All Trp2-vaccinated animals responded to peptide restimulation with a significant increase in IFNγ response achieved in rIL-21-treated aged mice compared to control PBS aged mice (Fig. 6D). Similarly, enhanced proliferation responses were

observed in the rIL-21-treated aged mice as evaluated by cell trace dilution overtime by flow cytometry (Fig. 6E) and compiled MFIs data (Fig. 6F). In sum, these data clearly show that rIL-21 preconditioning of aged mice leads to marked improvements in the control of B16 tumor growth following Trp2 vaccination.

## Discussion

Thymic involution deprives aged hosts from a competent immune system capable of effectively responding to vaccination and invading pathogens (Boehm & Swann, 2013). Although some aspects of age-related decline in T-cell responses reflect systemic changes, others are due to cell intrinsic defects (Haynes & Swain, 2006; Maue *et al.*, 2009; Tsukamoto *et al.*, 2009). We show in this report that administration of rIL-21 enhances thymopoiesis in aged mice through expansion of both the stromal and responsive thymocytes compartments without the induction of any apparent pathology in peripheral organs. Consequently, a competent peripheral lymphoid pool containing larger proportion of naïve $CD4^+$ and $CD8^+$ T cells ($CD62L^{hi}CD44^{lo}$) displaying potent effector functions in response to TCR stimulation was restored. This increase in the availability and potency of naïve T cells augmented the responsiveness of aged mice to Trp2 vaccination and accounts for their improved survival in response to B16 tumor challenge (Fig. S7).

T lymphopoiesis remains functional at older age albeit to a limited extent (Hale *et al.*, 2006). As the thymus lacks self-renewing progenitors, it heavily relies on sustained seeding with BM-derived CLPs and/or ETPs (Min *et al.*, 2004). Unfortunately, however, BM-derived progenitor numbers decline markedly with age due to increased apoptosis rates as well as reduced proliferative capacities (Min *et al.*, 2004). This in turn negatively impacts the delicate thymic stromal compartment, which is dependent on cross talk interactions with thymic progenitors for its sustained survival and morphogenesis (Shores *et al.*, 1991; Hollander *et al.*, 1995; van Ewijk *et al.*, 2000; Dudakov *et al.*, 2012). In this regard, two major points can be concluded based on observations made in rIL-21-treated aged mice. First, a report by Ozaki *et al.* previously demonstrated that IL-21 overexpression in WT young mice expands LSK cells *in vivo* (Ozaki *et al.*, 2006). Such effect is certainly beneficial as it can increase the pool of BM progenitor cells available for thymic migration. Although we did not directly assess the level of thymus-migrating BM progenitors, we confirmed that all LSK subpopulations in ageing mice express IL-21R but fail to proliferate in response to rIL-21 administration (Fig. S2). Such apparent discrepancy between our observations and those reported by Ozaki and colleagues may be explained by the lower dose or bioavailability of rIL-21 in comparison with *in vivo* overexpression. However, age-related changes in the functional properties of LSK cells should also be taken into account. While the frequency of LT-HSCs increases drastically in the BM of ageing mice, a decrease in the ST-HSC and the transiently reconstituting MPP populations occurs in parallel (confirmed in Fig. S2) (Rossi *et al.*, 2005). Such alterations are elemental to thymopoiesis as the ST-HSC and MPP fractions both lie upstream of CLP and contribute to lymphoid reconstitution (Rossi *et al.*, 2005). These observations are consistent with microarray analyses revealing the existence of systematic downregulation of LT-HSC-specific genes mediating lymphoid specification and function with ageing (Rossi *et al.*, 2005). This implies that rIL-21 can compensate for deficiencies affecting BM-derived progenitors by stimulating the expansion of progenitor cells such as ETPs that have already seeded the thymic compartment. Second, the increase in the absolute number of cTEC and mTEC subpopulations in rIL-21-treated aged mice cannot be mediated by a direct action of rIL-21 due to the absence of IL-21R on TEC surface (Rafei *et al.*, 2013a). The simplest interpretation of these results is that increased thymocyte numbers enhanced lympho-stromal interactions consequently promoting TECs expansion (Hollander *et al.*, 1995; van Ewijk *et al.*, 2000). Indeed, BM transplantation studies using $RAG^{null}$ mice as recipients revealed a central role played by developing thymocytes in the functional organization of thymic microenvironments (van Ewijk *et al.*, 2000). By first providing signals to cTECs, DN thymocytes initiate the creation of a functional 3D organized cortical microenvironment. Such restructuring facilitates the differentiation of DN thymocytes to DP and SP stages, which ends up improving the survival/expansion of the mTEC compartment (van Ewijk *et al.*, 2000). This is certainly a plausible explanation as the adult thymus contains nonsenescent progenitor stromal cells believed to be involved in TEC maintenance (Dumont-Lagace *et al.*, 2014). Thus, an involuted stroma possibly retains the capacity to regenerate in response to increased progenitor numbers. Under such context, the lack of rIL-21-induced thymopoiesis in younger mice may not be surprising as both thymic size and function are optimal at that age.

The concept that miR-181a acts as a rheostat for TCR signaling is not novel (Li *et al.*, 2007). Although expression of signaling molecules involved in TCR signaling is not influenced by age (Tamura *et al.*, 2000), cumulative homeostatic proliferation is believed to promote progressive loss of miR-181a in ageing naïve T cells overtime (Li *et al.*, 2012). Only a continuous stream of newly developed T cells capable of replenishing the peripheral T-cell compartment can compensate for such intrinsic defect. In accordance with these studies, we detected lower expression of miR-181a in T cells of aged mice compared to their younger counterparts, which was further reflected on their poor TCR responsiveness (Fig. 4). However, *in vivo* provision of exogenous rIL-21 reversed the miR-181a decrease through enhanced *de novo* generation of RTEs that have incorporated the peripheral compartment. RTE maturation thereafter led to an increase in mature naïve T cells, which were competent enough to effectively respond to Trp2 vaccination and trigger a substantial delay in B16 tumor growth (Figs 3, 5, and 6). This, however, does not preclude the possibility that rIL-21 administration to aged mice affects other preexisting immune cells in periphery. Additional studies involving thymectomized mice or adoptive transfer into athymic nude are required to confirm this hypothesis. In addition, there are data indicating that RTEs generated in aged hosts retain some intrinsic defects following TCR cross-linking (Clise-Dwyer *et al.*, 2007). If so, how can we interpret the improved effector functions observed in the rejuvenated T-cell pool of rIL-21-treated mice? Clise-Dwyer *et al.* demonstrated that RTEs generated either following transplantation of younger hosts with aged BM or after antibody-mediated depletion of peripheral T-cell in ageing hosts exhibit normal effector function in comparison with RTEs developed in untreated aged animals (Clise-Dwyer *et al.*, 2007). The authors explained this conundrum by suggesting that under lymphopenic conditions, levels of IL-7 are either elevated or competition for IL-7 is reduced resulting in rescued T-cell development. As stromal cells are the main producers of IL-7 in the thymus, an ageing thymic microenvironment with increased TECs turnover would indeed compromise RTEs number, quality, and function due to limited IL-7 availability. This highlights one of the distinctive thymopoiesis-stimulating properties of rIL-21 as it can raise the level of intrathymic IL-7 production (Fig. 1SE) most likely by indirectly mediating the expansion of the stromal compartment through enhanced thymocyte proliferation therefore resulting in the generation of defects-free RTEs.

Besides physiological ageing, contraction of the TCR repertoire is commonly observed in patients suffering from infections, cancers, or following BM transplantation (van den Brink *et al.*, 2004). There are currently no effective therapies capable of exerting a positive impact on broadening the spectrum of TCR. Studies involving IL-21R$^{-/-}$ mice clearly showed that IL-21 is dispensable for immune cell development as normal proportions of lymphocytes, monocytes, and granulocytes have been reported (Spolski & Leonard, 2014). Our data nevertheless suggest that rIL-21 administration to ageing hosts could have potent clinical uses related to its ability to promote the expansion of thymic progenitor cells, which can be further enhanced if combined with other thymostimulatory compounds.

## Experimental procedures

### Cell line and mice

The B16F0 mouse melanoma cell line was kindly provided by Dr. J. Galipeau (Atlanta, GA, USA). The RAG2p-GFP transgenic mice were kindly provided by Dr. M. Nussenzweig (Rockefeller University, New York, NY, USA). Female WT C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). To generate IL-21R$^{-/-}$ C57BL/6 mice, commercially available sperm was purchased from the MMRRC repository and used to fertilize female WT C57BL/6 mice. All mice were housed at the Institute for Research in Immunology and Cancer (IRIC) animal facility under specific pathogen-free conditions. All animal protocols were approved by the Animal Care Committee of Université de Montréal.

### Antibodies, cytokines and reagents

Mouse rIL-21 and granulocyte–macrophage colony-stimulating factor (rGM-CSF) were purchased from Peprotech (Rocky Hill, NJ, USA). All flow cytometry antibodies and Cytofix/Cytoperm Kits were purchased from BD Pharmingen (San Diego, CA, USA). The anti-SHP-2, PTPN-22, DUSP5/6, and β-actin antibodies used in Western blotting were purchased from Abcam (Cambridge, MA, USA). Quantikines were purchased from R&D System (Minneapolis, MN, USA). The CD3-CD28 beads, cell trace, and Trizol were purchased from Invitrogen (Burlington, ON, Canada). The cell Lytic M buffer and lipopolysaccharide (LPS) were purchased from Sigma (St-Louis, MO, USA). T-cell enrichment kits were purchased from StemCell Technologies (Vancouver, BC, Canada). Peptides were synthesized by GenScript (Piscataway, NJ, USA).

### Administration of rIL-21 and T-cell analyses

For IL-21 dosage establishment, WT young (2M), or aged (15M) female mice were IP-injected with rIL-21 in 200 μL PBS on a daily interval (total of five injections) in the first week. Control mice received equal volumes of PBS. All subsequent *in vivo* experiments were performed using rIL-21 at 50 μg kg$^{-1}$. Peripheral T-cell absolute numbers were obtained by combining the analysis of blood samples collected from treated RAG2p-GFP mice using the Scil-Vet ABC$^{+}$ hematological analyzer (to obtain absolute counts of lymphocytes) and flow cytometry (to obtain percentages of specific subpopulations).

### Enrichment of primary TECs

Primary TECs were enriched from thymic lobes of WT C57Bl/6 mice as previously described (Gray *et al.*, 2006).

### Western blots

Splenocytes were first isolated to sort total CD3$^{+}$ T cells. Whole T-cell extracts were obtained through using the Cell Lytic M reagent (Sigma). Extracts were separated by electrophoresis, transferred onto Hybond-ECL membrane, and probed using primary antibodies. Densitometry analysis was conducted by first imaging the chemiluminescent blots with the ImageQuant LAS 4000 imager (GE Healthcare Life Sciences, Mississauga, ON, Canada) followed by analysis using the ImageQuant TL software (GE Healthcare Life Sciences).

### Expression analysis of miR-181a

T cells were first sorted directly in 900 μL Trizol ($10^6$ cells per tube) and lysed followed by RNA extraction in Trizol reagent (Invitrogen). RNA purification was further carried out using the RNA extraction kit (QIAgen, Toronto, On, Canada). Reverse transcription was performed using the High Capacity cDNA reverse transcription kit and qPCR was performed with a 7900HT Fast Real-Time PCR system at IRIC's genomic core facility. Target gene values were normalized to endogenous control *Gapdh*.

### Intracellular staining

For analysis of phosphorylated (p)Lck, ZAP-70, and ERK, isolated splenocytes were first stimulated *in vitro* using CD3-CD28 Dynabeads (Invitrogen) for 5 min, then washed and surface stained for CD4 and CD8 prior to intracellular staining according to manufacturer's instructions.

### Histological analyses

Chosen organs were harvested from treated mice, fixed in 10% formalin before mounting in paraffin. Sections were then stained with hematoxylin and eosin, then scanned using the NanoZoomer Digital Pathology system and NPD.scan 2.3.4 software (Hamamatsu, Hamamatsu city, Japan).

### DC vaccination and tumor challenge

To generate DCs for vaccination, BM cells were extracted from tibia/femur bones of 8-week-old male mice and plated in 10-cm nontissue culture-treated Petri dishes containing 10 mL of complete RPMI 1640 medium (0.048 mmol L$^{-1}$ β-mercaptoethanol, 2 mmol L$^{-1}$ L-glutamine, 10% fetal bovine serum, 2 mmol L$^{-1}$ penicillin–streptavidin) supplemented with 10 ng mL$^{-1}$ rGM-CSF (Peprotech). The media were changed at days 3 and 6 of culture. To induce DC maturation, 1 μg mL$^{-1}$ LPS (Sigma) was added at day 8 for 24 h. Following confirmation of a mature phenotype (>80% of adherent cells were CD80$^{+}$ CD86$^{+}$ MHCI$^{+}$ MHCII$^{+}$), DCs were pulsed with OVA- or Trp2-derived peptides (GenScript) for 4 h, then harvested for intravenous vaccination. Two weeks following the last boost, mice were SC-challenged with $5 \times 10^5$ B16 tumor cells. Tumor volume was measured as [(length × width$^2$)/2], and mice were sacrificed when the tumor volume reached 2000 mm$^3$.

### Proliferation and cytokine measurements

For all assays conducted using nonvaccinated mice, total or fractionated T cells were first isolated by negative selection, then plated at

$10^5$ cells well$^{-1}$ in 96-well plates. To trigger proliferation/activation, T cells were stimulated with CD3-CD28 Dynabeads in a 1:1 ratio. For *in vitro* recall responses following vaccination, splenocytes were pulsed with 1 μg mL$^{-1}$ Trp2 peptide. Cells or supernatants were collected 48 h poststimulation for further analyses.

### Statistical analyses

*P*-values were calculated using the ANOVA and log-rank statistical test where applicable. Log-rank testing was performed using software available at the Walter and Eliza Hall Institute Web site (http://bioinf.wehi.edu.au/software/russell/logrank/).

## Acknowledgments

We are grateful to Danièle Gagné and Serge Sénéchal (for sorting and flow cytometry assistance), Raphaëlle Lambert (for qPCR studies), and to the staff of the IRIC histology and animal care facility for their assistance. This work was supported by a Cancer Research Society (CRS) operating grant. Moutih Rafei holds a Fonds de la Recherche en Santé du Québec Junior 1 award.

## Author contributions

EAC designed most of the study, carried out experiments, analyzed the data, prepared the figures, and wrote the first draft of the manuscript. AT, SP, RK, and SZ performed some experiments and contributed to data analysis and manuscript preparation. MR designed the study, discussed the results with all authors, and wrote the manuscript. The authors declare that they have no conflict of interest.

## References

Ahmed M, Lanzer KG, Yager EJ, Adams PS, Johnson LL, Blackman MA (2009) Clonal expansions and loss of receptor diversity in the naive CD8 T cell repertoire of aged mice. *J. Immunol.* **182**, 784–792.

Attridge K, Wang CJ, Wardzinski L, Kenefeck R, Chamberlain JL, Manzotti C, Kopf M, Walker LS (2012) IL-21 inhibits T cell IL-2 production and impairs Treg homeostasis. *Blood* **119**, 4656–4664.

Berkley AM, Hendricks DW, Simmons KB, Fink PJ (2013) Recent thymic emigrants and mature naive T cells exhibit differential DNA methylation at key cytokine loci. *J. Immunol.* **190**, 6180–6186.

Boehm T, Swann JB (2013) Thymus involution and regeneration: two sides of the same coin? *Nature reviews. Immunology* **13**, 831–838.

Boursalian TE, Golob J, Soper DM, Cooper CJ, Fink PJ (2004) Continued maturation of thymic emigrants in the periphery. *Nat. Immunol.* **5**, 418–425.

van den Brink MR, Alpdogan O, Boyd RL (2004) Strategies to enhance T-cell reconstitution in immunocompromised patients. *Nat. Rev. Immunol.* **4**, 856–867.

Clise-Dwyer K, Huston GE, Buck AL, Duso DK, Swain SL (2007) Environmental and intrinsic factors lead to antigen unresponsiveness in CD4(+) recent thymic emigrants from aged mice. *J. Immunol.* **178**, 1321–1331.

Decman V, Laidlaw BJ, Doering TA, Leng J, Ertl HC, Goldstein DR, Wherry EJ (2012) Defective CD8 T cell responses in aged mice are due to quantitative and qualitative changes in virus-specific precursors. *J. Immunol.* **188**, 1933–1941.

Dudakov JA, Hanash AM, Jenq RR, Young LF, Ghosh A, Singer NV, West ML, Smith OM, Holland AM, Tsai JJ, Boyd RL, van den Brink MR (2012) Interleukin-22 drives endogenous thymic regeneration in mice. *Science* **336**, 91–95.

Dumont-Lagace M, Brochu S, St-Pierre C, Perreault C (2014) Adult thymic epithelium contains nonsenescent label-retaining cells. *J. Immunol.* **192**, 2219–2226.

Eaton SM, Burns EM, Kusser K, Randall TD, Haynes L (2004) Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses. *J. Exp. Med.* **200**, 1613–1622.

van Ewijk W, Hollander G, Terhorst C, Wang B (2000) Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. *Development* **127**, 1583–1591.

Garcia GG, Miller RA (1997) Differential tyrosine phosphorylation of zeta chain dimers in mouse CD4 T lymphocytes: effect of age. *Cell. Immunol.* **175**, 51–57.

Garcia GG, Miller RA (2001) Single-cell analyses reveal two defects in peptide-specific activation of naive T cells from aged mice. *J. Immunol.* **166**, 3151–3157.

Garcia GG, Miller RA (2002) Age-dependent defects in TCR-triggered cytoskeletal rearrangement in CD4 + T cells. *J. Immunol.* **169**, 5021–5027.

Gray DH, Seach N, Ueno T, Milton MK, Liston A, Lew AM, Goodnow CC, Boyd RL (2006) Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. *Blood* **108**, 3777–3785.

Hale JS, Boursalian TE, Turk GL, Fink PJ (2006) Thymic output in aged mice. *Proc. Natl Acad. Sci. USA* **103**, 8447–8452.

Haynes L, Swain SL (2006) Why aging T cells fail: implications for vaccination. *Immunity* **24**, 663–666.

Hollander GA, Wang B, Nichogiannopoulou A, Platenburg PP, van Ewijk W, Burakoff SJ, Gutierrez-Ramos JC, Terhorst C (1995) Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. *Nature* **373**, 350–353.

Houston EG Jr, Nechanitzky R, Fink PJ (2008) Cutting edge: contact with secondary lymphoid organs drives postthymic T cell maturation. *J. Immunol.* **181**, 5213–5217.

Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, Oukka M, Kuchroo VK (2007) IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* **448**, 484–487.

Lages CS, Suffia I, Velilla PA, Huang B, Warshaw G, Hildeman DA, Belkaid Y, Chougnet C (2008) Functional regulatory T cells accumulate in aged hosts and promote chronic infectious disease reactivation. *J. Immunol.* **181**, 1835–1848.

Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, Braich R, Manoharan M, Soutschek J, Skare P, Klein LO, Davis MM, Chen CZ (2007) miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* **129**, 147–161.

Li G, Yu M, Lee WW, Tsang M, Krishnan E, Weyand CM, Goronzy JJ (2012) Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. *Nat. Med.* **18**, 1518–1524.

Lynch HE, Goldberg GL, Chidgey A, Van den Brink MR, Boyd R, Sempowski GD (2009) Thymic involution and immune reconstitution. *Trends Immunol.* **30**, 366–373.

Maue AC, Yager EJ, Swain SL, Woodland DL, Blackman MA, Haynes L (2009) T-cell immunosenescence: lessons learned from mouse models of aging. *Trends Immunol.* **30**, 301–305.

Min H, Montecino-Rodriguez E, Dorshkind K (2004) Reduction in the developmental potential of intrathymic T cell progenitors with age. *J. Immunol.* **173**, 245–250.

Monroe RJ, Seidl KJ, Gaertner F, Han S, Chen F, Sekiguchi J, Wang J, Ferrini R, Davidson L, Kelsoe G, Alt FW (1999) RAG2:GFP knockin mice reveal novel aspects of RAG2 expression in primary and peripheral lymphoid tissues. *Immunity* **11**, 201–212.

Ozaki K, Hishiya A, Hatanaka K, Nakajima H, Wang G, Hwu P, Kitamura T, Ozawa K, Leonard WJ, Nosaka T (2006) Overexpression of interleukin 21 induces expansion of hematopoietic progenitor cells. *Int. J. Hematol.* **84**, 224–230.

Parkhurst MR, Fitzgerald EB, Southwood S, Sette A, Rosenberg SA, Kawakami Y (1998) Identification of a shared HLA-A*0201-restricted T-cell epitope from the melanoma antigen tyrosinase-related protein 2 (TRP2). *Cancer Res.* **58**, 4895–4901.

Peluso I, Fantini MC, Fina D, Caruso R, Boirivant M, MacDonald TT, Pallone F, Monteleone G (2007) IL-21 counteracts the regulatory T cell-mediated suppression of human CD4 + T lymphocytes. *J. Immunol.* **178**, 732–739.

Rafei M, Hardy MP, Williams P, Vanegas JR, Forner KA, Dulude G, Labrecque N, Galipeau J, Perreault C (2011) Development and function of innate polyclonal TCRalphabeta+ CD8 + thymocytes. *J. Immunol.* **187**, 3133–3144.

Rafei M, Dumont-Lagace M, Rouette A, Perreault C (2013a) Interleukin-21 accelerates thymic recovery from glucocorticoid-induced atrophy. *PLoS ONE* **8**, e72801.

Rafei M, Rouette A, Brochu S, Vanegas JR, Perreault C (2013b) Differential effects of gammac cytokines on postselection differentiation of CD8 thymocytes. *Blood* **121**, 107–117.

Rossi DJ, Bryder D, Zahn JM, Ahlenius H, Sonu R, Wagers AJ, Weissman IL (2005) Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proc. Natl Acad. Sci. USA* **102**, 9194–9199.

Shores EW, Van Ewijk W, Singer A (1991) Disorganization and restoration of thymic medullary epithelial cells in T cell receptor-negative scid mice: evidence

that receptor-bearing lymphocytes influence maturation of the thymic microenvironment. *Eur. J. Immunol.* **21**, 1657–1661.

Spolski R, Leonard WJ (2014) Interleukin-21: a double-edged sword with therapeutic potential. *Nat. Rev. Drug Discovery* **13**, 379–395.

Tamir A, Eisenbraun MD, Garcia GG, Miller RA (2000) Age-dependent alterations in the assembly of signal transduction complexes at the site of T cell/APC interaction. *J. Immunol.* **165**, 1243–1251.

Tamura T, Kunimatsu T, Yee ST, Igarashi O, Utsuyama M, Tanaka S, Miyazaki S, Hirokawa K, Nariuchi H (2000) Molecular mechanism of the impairment in activation signal transduction in CD4(+) T cells from old mice. *Int. Immunol.* **12**, 1205–1215.

Tough DF, Sprent J (1994) Turnover of naive- and memory-phenotype T cells. *J. Exp. Med.* **179**, 1127–1135.

Tsukamoto H, Clise-Dwyer K, Huston GE, Duso DK, Buck AL, Johnson LL, Haynes L, Swain SL (2009) Age-associated increase in lifespan of naive CD4 T cells contributes to T-cell homeostasis but facilitates development of functional defects. *Proc. Natl Acad. Sci. USA* **106**, 18333–18338.

Yager EJ, Ahmed M, Lanzer K, Randall TD, Woodland DL, Blackman MA (2008) Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *J. Exp. Med.* **205**, 711–723.

Yu W, Nagaoka H, Jankovic M, Misulovin Z, Suh H, Rolink A, Melchers F, Meffre E, Nussenzweig MC (1999) Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. *Nature* **400**, 682–687.

Zanni F, Vescovini R, Biasini C, Fagnoni F, Zanlari L, Telera A, Di Pede P, Passeri G, Pedrazzoni M, Passeri M, Franceschi C, Sansoni P (2003) Marked increase with age of type 1 cytokines within memory and effector/cytotoxic CD8+ T cells in humans: a contribution to understand the relationship between inflammation and immunosenescence. *Exp. Gerontol.* **38**, 981–987.

Zhang X, Fujii H, Kishimoto H, LeRoy E, Surh CD, Sprent J (2002) Aging leads to disturbed homeostasis of memory phenotype CD8(+) cells. *J. Exp. Med.* **195**, 283–293.

## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

**Fig. S1** Analysis of thymic $GFP^+$ content, absolute counts and IL-7 production following rIL-21 administration.

**Fig. S2** Administration of rIL-21 to aged mice has no beneficial effect on the BM compartment.

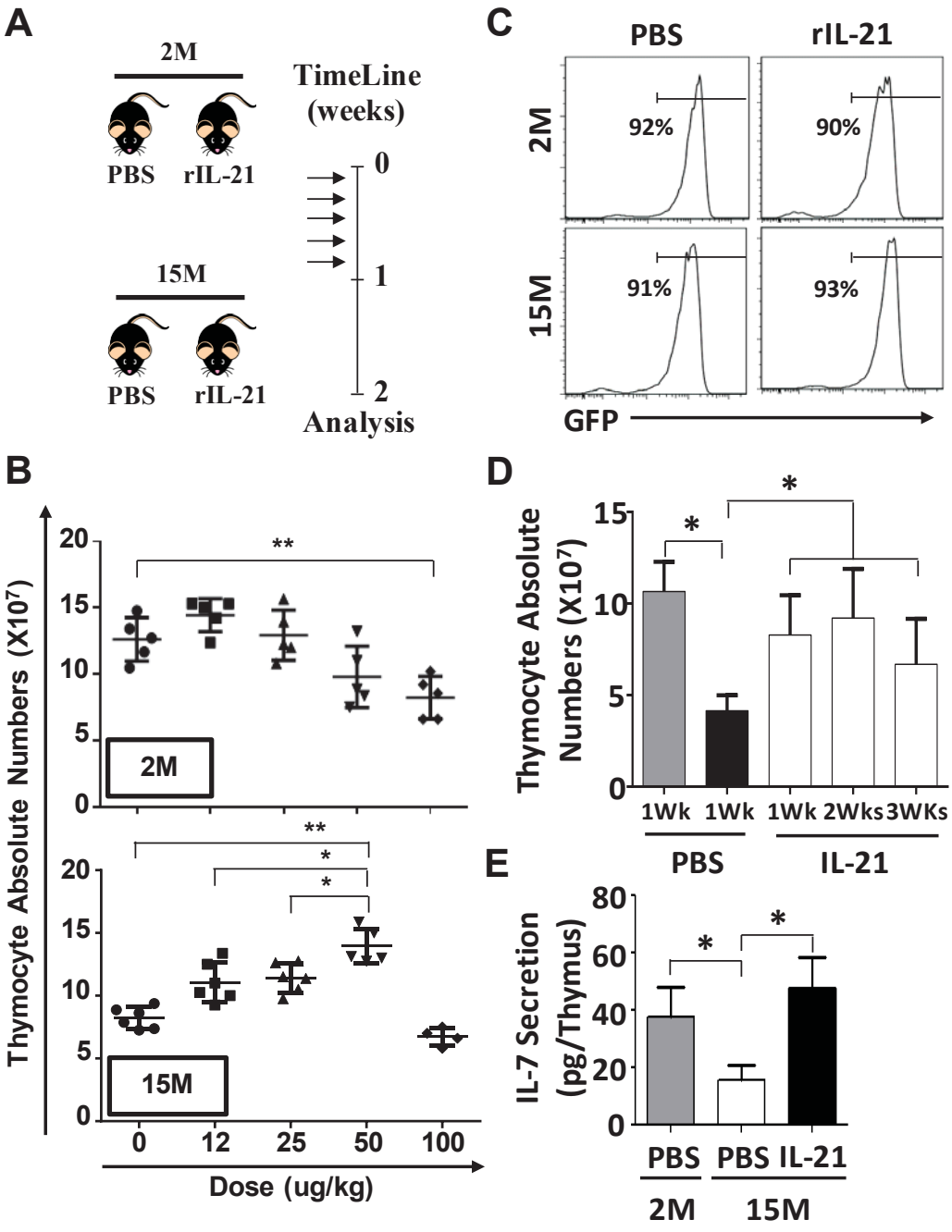
**Fig. S3** Assessment of TCR repertoire diversity following rIL-21 administration to young mice.

**Fig. S4** Transcription factors quantification in T cells derived from treated mice.

**Fig. S5** Assessment of autoimmune signs following rIL-21 administration.

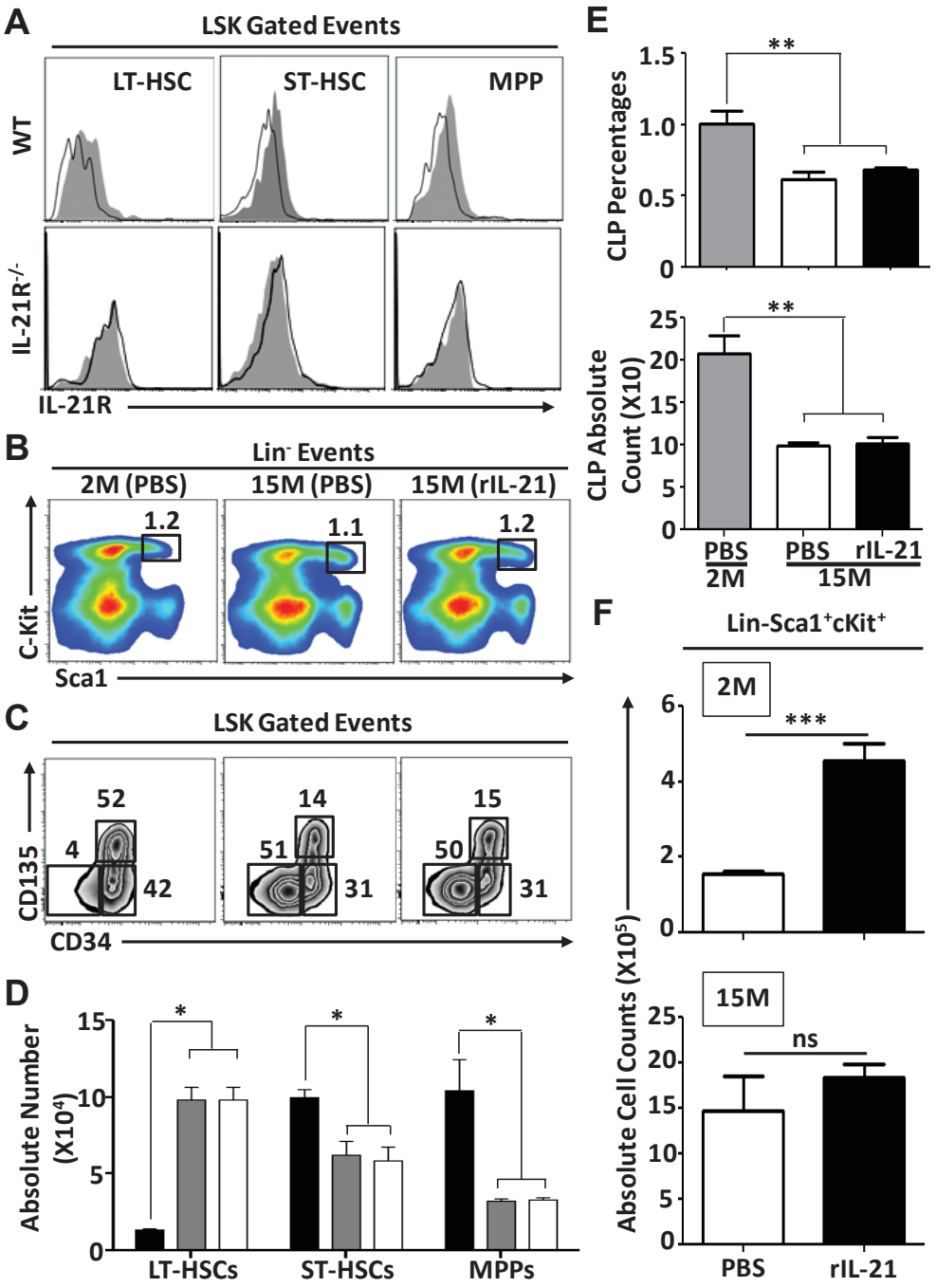
**Fig. S6** Generation and characterization of BM-derived DC for vaccination.

**Fig. S7** rIL-21 administration to aged mice increases thymic output leading to improved immune functions.



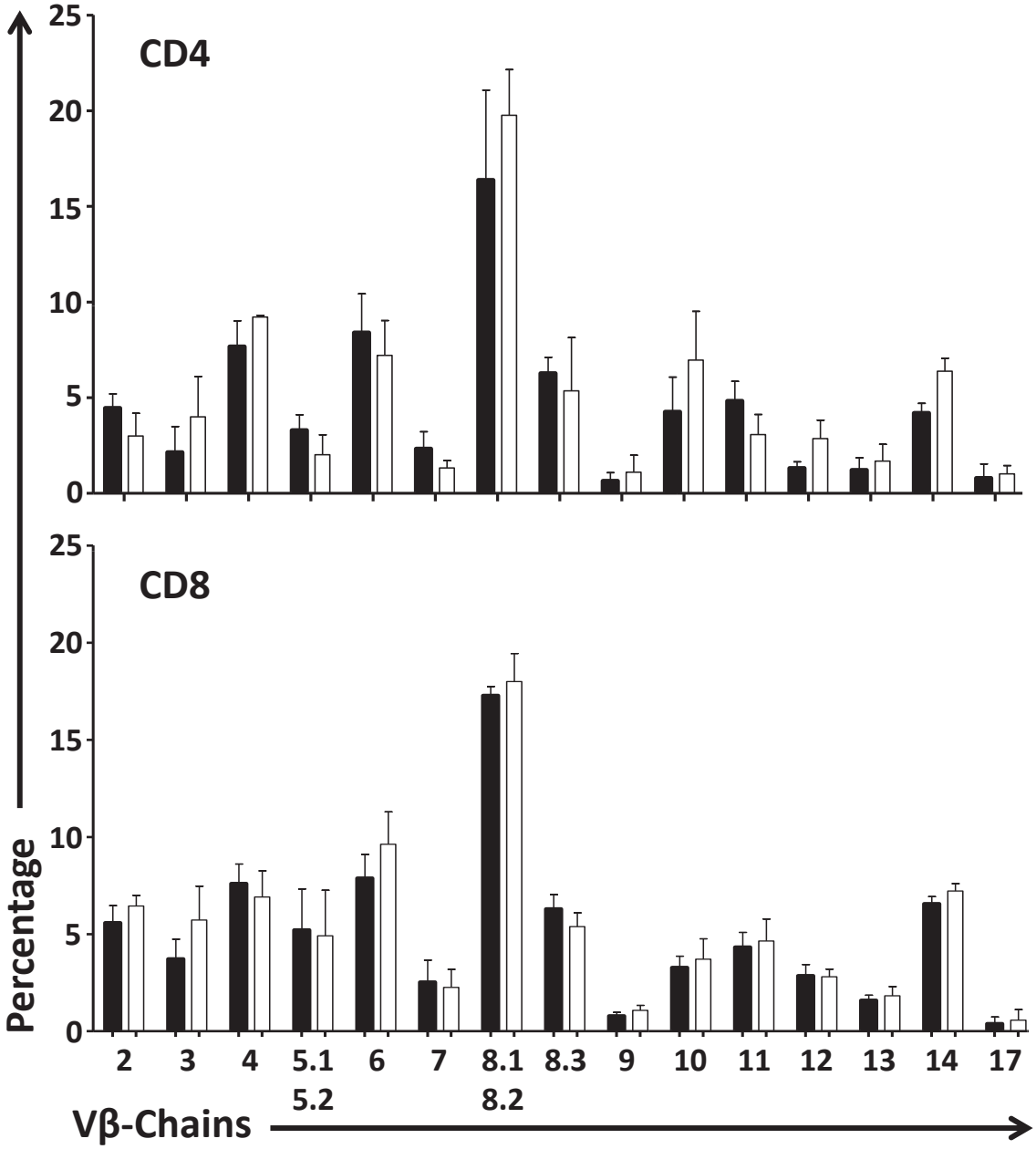

**Supplementary Figure S1: Analysis of thymic GFP$^+$ content, absolute counts and IL-7 production following rIL-21 administration.**

A) Schematic diagram representing the strategy and group of mice used to identify the optimal dose of rIL-21 capable of enhancing *de novo* thymopoiesis. Thymi were collected and analyzed one week following the last rIL-21 administration. B) Total thymocyte count following administration of ascending doses of rIL-21 to young (2M) or aged (15M) WT female C57BL/6 mice. C) Representative flow-cytometry analysis of thymic GFP content. D) Absolute counts of total thymocytes in 2M (PBS□) or 15M (PBS■) 1 week post-PBS treatment versus 15M (rIL-21 □) at 1, 2, and 3 weeks post-rIL-21 treatment. E) ELISA-based quantification of IL-7 secretion by thymus one week post-treatment. All data are representative of three independent experiments (n = 5/group with *p<0.05).



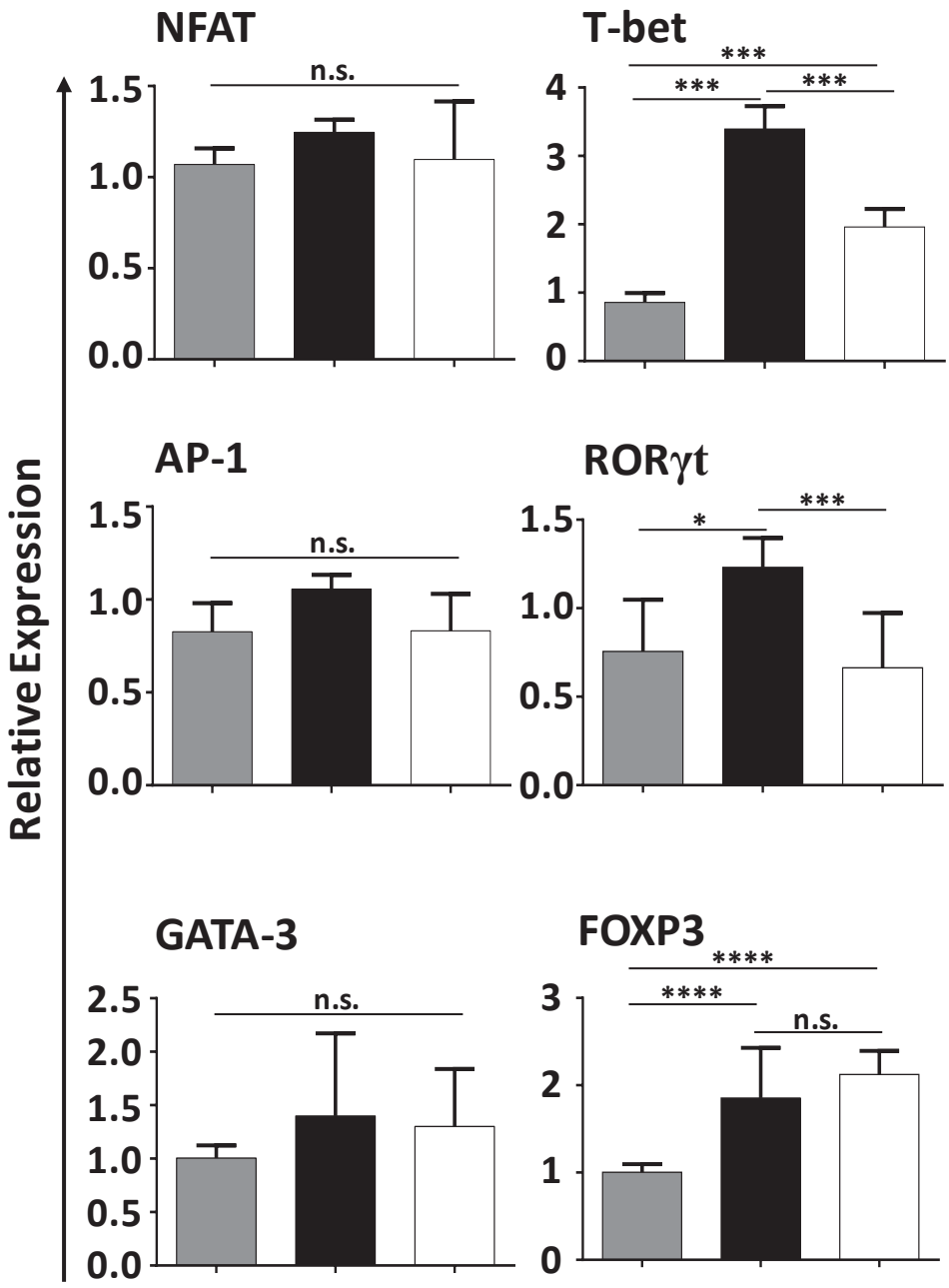

**Supplementary Figure S2: Administration of rIL-21 to aged mice has no beneficial effect on the BM compartment.**

A) Representative flow-cytometry analysis of IL-21R expression on the surface of LT-, ST-HSCs and MPPs derived from WT (top panels) or IL-21R$^{-/-}$ mice (lower panels). Filled histograms represent IL-21R expression. B-C) Percentages of total LSK cells or LT-, ST-HSCs as well as MPPs as depicted by flow-cytometry. D) Absolute counts of LSK sub-populations in 2M (PBS■), 15M (PBS□), and 15M (rIL-21□) aged mice. E) CLP percentage and absolute counts in 2M (PBS□), 15M (PBS□), and 15M (rIL-21■) aged mice. F) Absolute counts of LSK derived from young or aged mice following *in vitro* PBS or rIL-21 treatment. All data are representative of three independent experiments (n=5/group with *p<0.05; **p<0.01, and ***p<0.001).



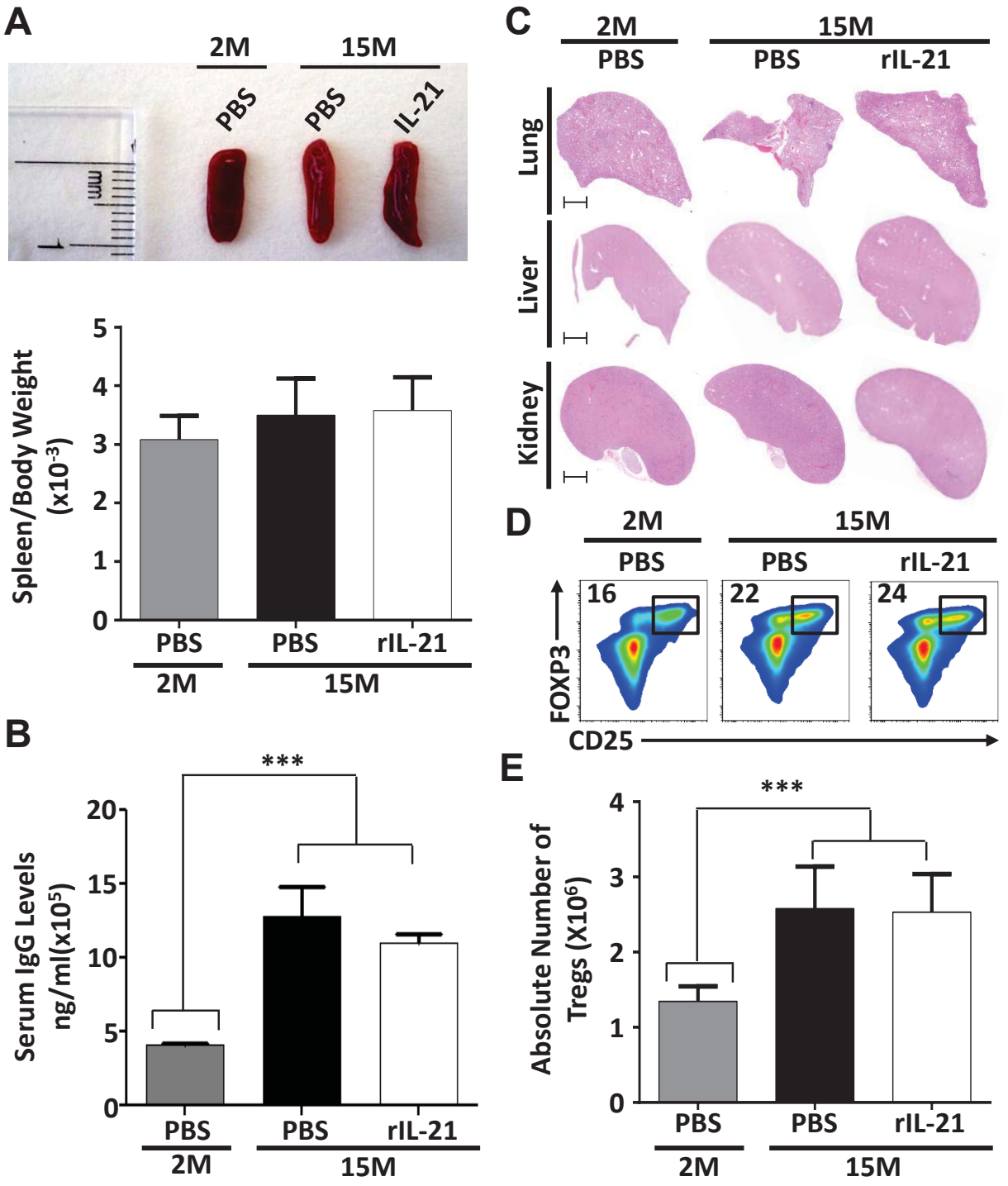

**Supplementary figure S3. Assessment of TCR repertoire diversity following rIL-21 administration to young mice**.

Flow-cytometry analysis of 15 TCRVβ-chains using spleen-derived $CD4^+$ (top panel) or $CD8^+$ (lower panel) T cells from young 2M old mice treated with PBS (black bars) or rIL-21 (white bard). No significant differences were depicted between both groups. All data are representative of three independent experiments (n = 5/group).



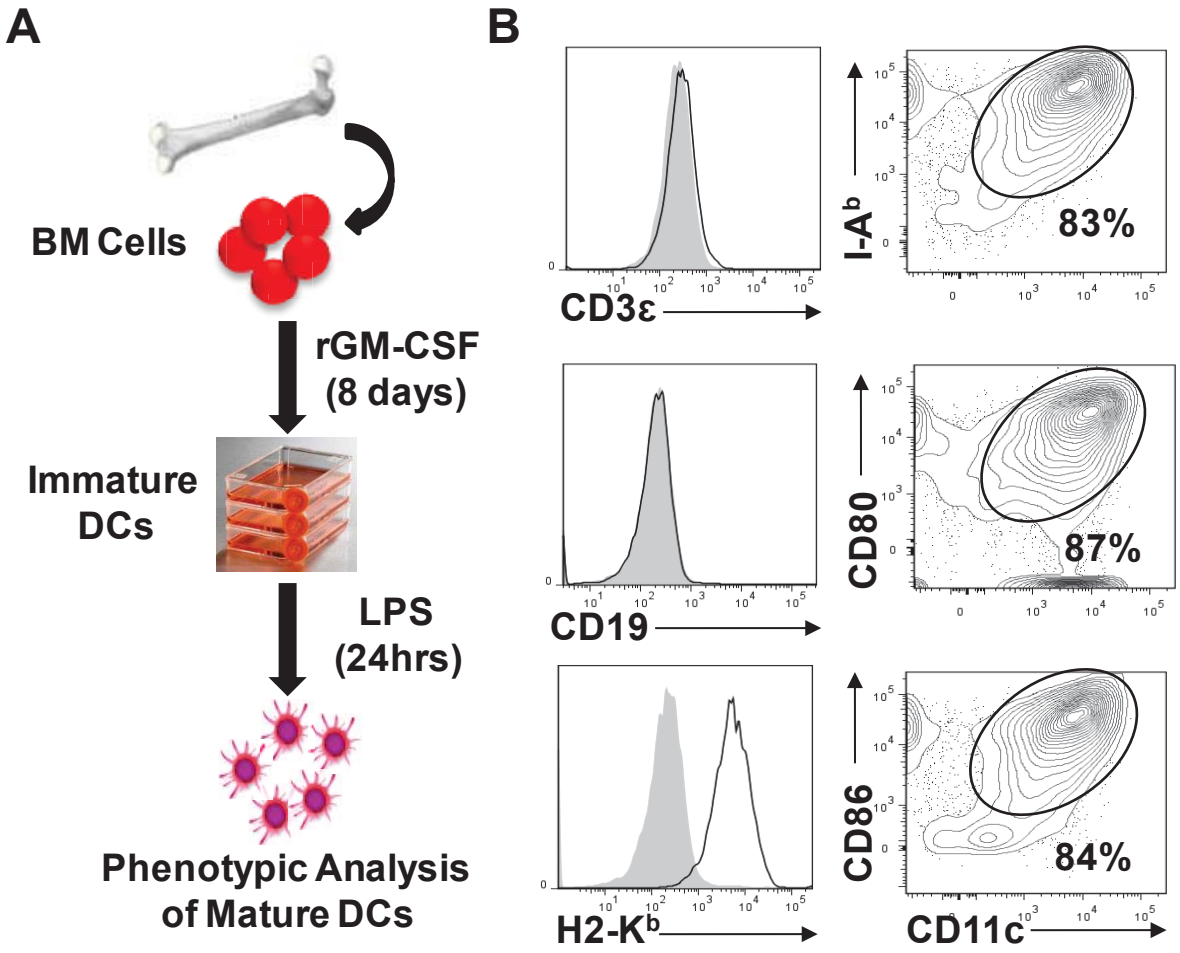

**Supplementary Figure S4: Transcription factors quantification in T cells derived from treated mice.**

T cells derived from treated mice were isolated then lysed to extract total mRNA. qPCR analyses were then conducted to quantify the expression of targeted genes relative to endogenous controls. Groups are displayed as: 2M (PBS□); 15M (PBS■); and 15M (rIL-21□) aged mice. All data are representative of three independent experiments (n=5/group with *p<0.05, ***p<0.001, and ****p<0.0001).



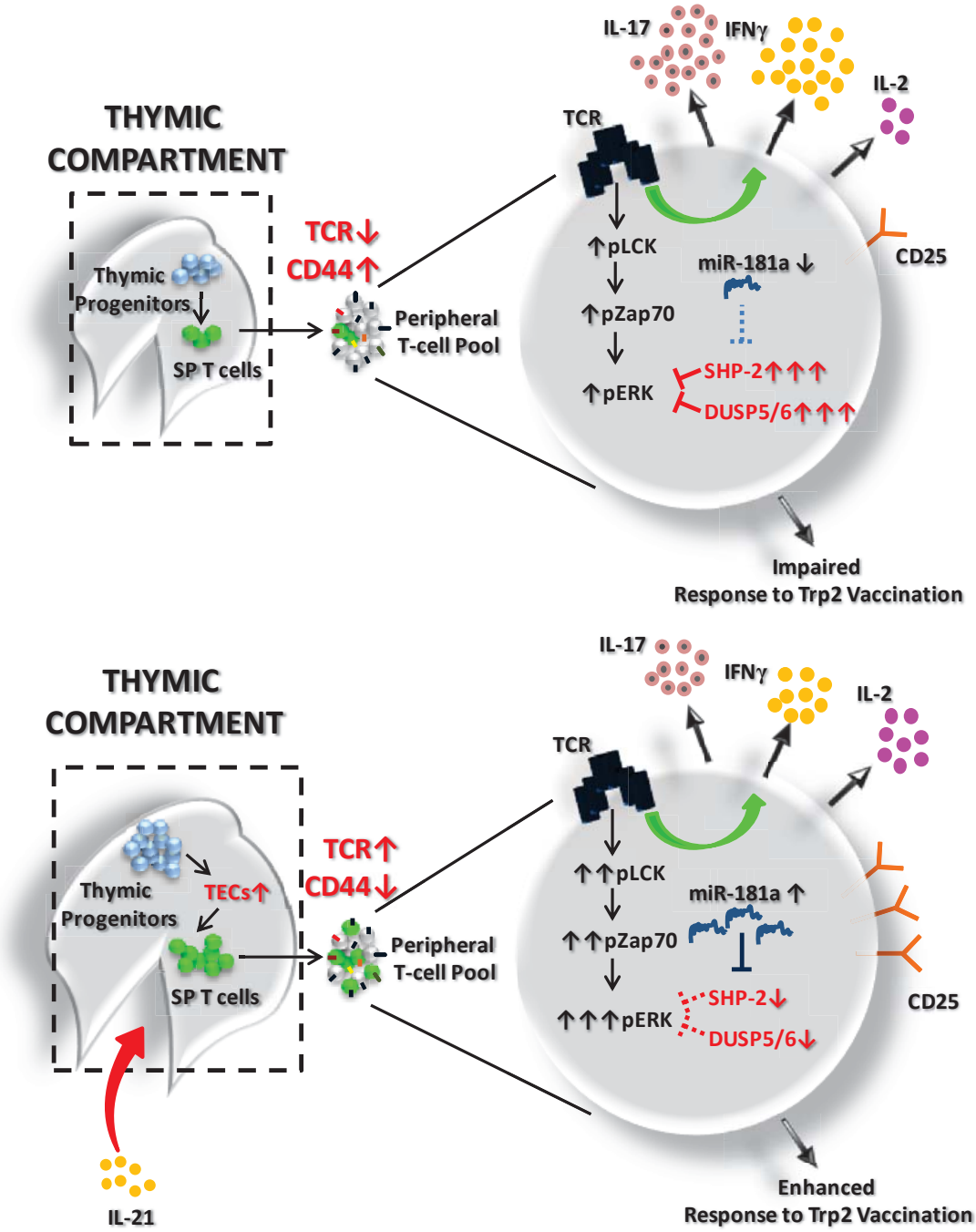

**Supplementary Figure S5: Assessment of autoimmune signs following rIL-21 administration.**

A) A representative photograph of spleens and their relative spleen to body weight derived from treated mice. B) Serum IgG levels quantified by ELISA. C) Hematoxylin-eosin staining of formalin-fixed sections of lung, liver and kidney of young, PBS- or rIL-21-treated aged mice. Scale bars represent 2 mm. D) Representative flow-cytometry analysis of $CD4^+CD25^+$ $T_{regs}$ in all experimental groups. We pre-gated on $CD4^+$ T cells prior to $CD25^+$ (cell surface) and FOXP3 (intracellular) analysis. E) Quantification of $CD4^+CD25^+$ $T_{regs}$ according to flow-cytometry analysis and splenocyte counts. All data are representative of three independent experiments (n = 5/group with ***p<0.001).




**Supplementary Figure S6: Generation and characterisation of BM-derived DC for vaccination.**

A) To generate BM-derived DCs, femur and tibias of male C57BL/6 mice where flushed to collect total nucleated cells, which were then plated for 8 days with rGM-CSF (10ng/ml). LPS was added to stimulate DC maturation at day 9. B) Representative flow-cytometry analysis of mature DC phenotype (>80% of DCs were $CD11c^{+}CD80^{+}CD86^{+}H2\text{-}K^{b+}I\text{-}A^{b+}$) using all non-adherent cells collected at day 10.




**Supplementary Figure S7: rIL-21 administration to aged mice increases thymic output leading to improved immune functions.**

In aging mice, thymopoiesis is diminished causing attrition of the TCR repertoire and accumulation of peripheral CD44$^{hi}$ T cells. In parallel, increased post-thymic life span leads to accumulated SHP-2 and DUSP5/6 due to decreased expression of miR-181a. As such, TCR stimulation of aging naïve T cells is weakened leading to lower CD25 expression along with poor secretion of IL-2 and thus, impaired immune functions. When given to aged mice, rIL-21 promotes thymopoiesis leading to increased T-cell output and improved TCR diversity. As RTEs increase the proportion of naïve T cells expressing higher level of miR-181a, SHP-2 and DUSP5/6 levels diminish allowing better T-cell responses translating into potent immunity.