The Role of the Sphingosine-1-Phosphate Pathway in Graft-versus-Host disease (GVHD) and T-cell Regeneration in murine allogeneic Hematopoietic Stem Cell Transplantation

Inauguraldissertation
zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Madeleine Vollmer
aus Basel, Schweiz

Basel, 2018

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von

Prof. Stephan Krähenbühl
PD Dr. Werner Krenger
Prof. Christoph Hess

Basel, den 26. Juni 2018

Prof. Dr. Martin Spiess
Dekan

Table of Contents

1. Abstract

The success of allogeneic hematopoietic stem cell transplantation (allo-HSCT) is limited by the complication of acute graft-versus-host disease (aGVHD) which may develop to some degree in up to 80% of patients. Unfortunately, aGVHD and the immunosuppression needed to control disease slow down posttransplantation immune regeneration and consequently also hamper anti-tumor immunity. Tipping the balance towards efficient immune reconstitution but against the development of aGVHD remains a major goal in allo-HSCT. Furthermore, in clinical allo-HSCT, the development of aGVHD (that is initiated by alloreactive donor T cells) predisposes to chronic GVHD (cGVHD) with autoimmune manifestations. It is currently unclear, however, how autoimmunity is linked to antecedent alloimmunity. Using murine models of allo-HSCT, I could show in a collaborative effort that autoreactive T cells can be generated *de novo* in the host thymus, implying an impairment in self-tolerance induction as a consequence of aGVHD. As a possible mechanism, we have published that loss of medullary thymic epithelium expressing the autoimmune regulator ($Aire^{+}mTEC^{high}$) was essential for failure to clonally delete self-reactive T cells. Our data therefore indicated that functional compromise of the mTEC compartment links alloimmunity to the development of autoimmunity during cGVHD. As a direct consequence of this work, I aimed to test in the second part of my PhD thesis project whether continuous blockade of donor T-cell trafficking from activation sites in secondary lymphoid organs (SLO) would prevent thymic injury and hence prevent the emergence of autoreactive T cells. Using different murine allo-HSCT models, I analyzed the effects of sphingosine-1-phosphate (S1P) pathway interference with the highly specific synthetic sphingosine-1-phosphate receptor 1 ($S1PR_1$) agonist KRP203. I found that prophylactic but not therapeutic $S1PR_1$ agonism reduced donor T-cell migration to the host thymus, thus significantly attenuating thymic aGVHD. Moreover, prophylactic KRP203 administration was found to allow for normal intrathymic T-cell maturation in the absence and presence of aGVHD. In consequence, the $Aire^{+}mTEC^{high}$ pool remained normal. Maintenance of the TEC compartment was indeed associated with the emergence of lower numbers of thymus-dependent autoreactive T cells in the periphery. Lastly, my data confirmed that $S1PR_1$ receptor agonism maintains the capacity to reject hematopoietic tumors that are retained in these sites. The present work closed gaps in knowledge with regard to the action profiles of $S1PR_1$ agonism on two separate parameters that govern transplant outcome, i.e. posttransplantation T-cell neogenesis and anti-tumor immunity. It may hence accelerate clinical trials and the definitive implementation of $S1PR_1$ receptor agonism as a principle for the prevention of aGVHD where the unmet medical need is high.

2. Introduction

2.1 Hematopoietic Stem Cell Transplantation (HSCT)

2.1.1 Current status of HSCT

Hematopoietic stem cell transplantation (HSCT) is a preeminent therapy for certain hematological and non-hematological disorders. Worldwide, the one millionth HSCT was announced in 2013, and in Europe more than 40'000 transplants were recorded in 2016.[1,2] Transferred HSC are obtained either from the patient itself (autologous), or from a genetically non-identical donor (allogeneic). The probability for a sibling being a compatible donor (human leukocyte antigen (HLA)-identical) is 25%.[3] If there is no suitable sibling or family member, patients mostly receive HSC from HLA-identical but unrelated donors or from donors that are HLA-haploidentical (haplo-ID).[4-6] There are several sources from which HSC can be obtained from the donor. The bone marrow (BM) punctuation was the conventional way to isolate HSC. Higher doses of HSC can now be harvested from the peripheral blood (PB) by mobilizing HSC from the BM with the help of the cytokine granulocyte-colony stimulating factor (G-CSF) in a procedure called peripheral HSC apheresis.[7,8] HSC can also be isolated from cord blood but the recovery is small.[9-11] In Europe, allogeneic HSCT (allo-HSCT) is performed in 43% of all transplanted patients and is the favored therapy for acute myeloid leukemia (AML, a malignant disease which constitutes 36% of all indications for which an allo-HSCT is done), acute lymphoblastic leukemia (ALL, 16%) and non-Hodgkin lymphoma (NHL, 8%). Allo-HSCT currently represents the only curative treatment for patients with myelodysplastic syndrome and myeloproliferative neoplasm (MDS/MPN, 15%) so far.[2,12] Other indications for an allo-HSCT are anemia, severe combined immune deficiencies (SCID) or metabolic diseases, as illustrated in Figure 1.[2,13]

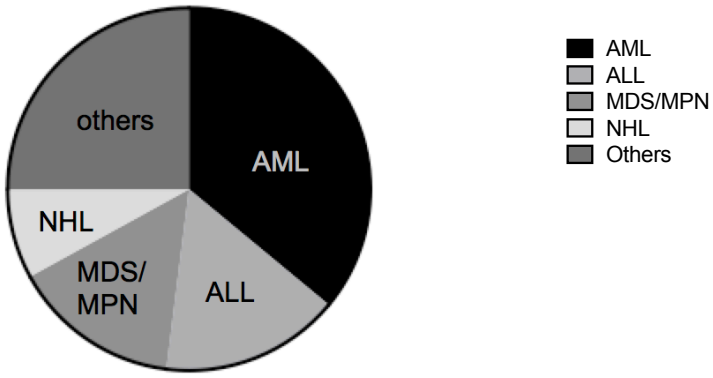


Figure 1: Relative proportions of disease indications for an allo-HSCT in Europe in 2014. *Acute myeloid leukemia (AML, 36%), acute lymphoblastic leukemia (ALL, 16%), non-Hodgkin lymphoma (NHL, 8%), myelodysplastic syndrome & myeloproliferative neoplasm (MDS/MPN 15%). Illustration is adapted from Passweg J. et al., Bone marrow transplantation, 2016.*[2]

2.1.2 Principles and clinical outcomes of allo-HSCT

Eligible patients for an allo-HSCT first have to undergo preparative conditioning regimens in the form of chemotherapy and/or total body irradiation (TBI).[14,15] The objectives are to reduce the bulk of malignant cells in instances where allo-HSCT is used for cancer treatment, decrease of the risk of graft rejection via general host immune suppression, and improve the engraftment of donor HSC by evacuating the host marrow. As a side effect the preconditioning also invariably compromises natural and adaptive immune responses of HSCT recipients, predisposing them to infections. This complication contributes to poor clinical outcome post-HSCT (see below). The classification of conditioning regimen intensity is based on the agents, doses and schedules used. According to the Center for International Blood and Marrow Transplant Research (CIBMTR), myeloablative conditioning regimens are defined as regimens with TBI of ≥500cGy, single fractionated doses of ≥800cGy, busulfan doses of >9mg/kg, or melphalan doses of >150mg/m^2 given as single agents or in combination with other drugs. Reduced-intensity conditioning (RIC) regimens are defined as regimens with lower doses of TBI and fractionated radiation therapy as well as lower doses of busulfan and melphalan than those used to define a myeloablative conditioning regimen.[16] Both, type of conditioning and intensity, depend on the age and health status of the patient. The concept of RIC makes the allo-HSCT procedure safer and is often preferred over myeloablative regimens in elderly patients.[17]

The subsequent stem cell transplantation then serves two purposes: I) to rescue the depleted host stem cell compartment with donor HSC which ultimately generate a new donor-derived complete hematopoietic system and II) to exploit the immunotherapeutic effect of anti-tumor activity of

infused mature donor T cells present in the donor graft (i.e. GVT, graft-versus tumor effect). These cells recognize tumor-associated and host allo-antigens.[18-20]

A successful clinical outcome of HSCT is determined by a functional immune competence, a long-term disease-free survival and the absence of transplant-related toxicities (TRT) stemming from graft-versus host disease (GVHD) (see below) and from conditioning (Figure 2). Within the first weeks after HSCT, there is no fully reconstituted immune system yet. The longer this phase persists, the higher is the risk to develop fungal and viral infections and to suffer from tumor relapse.[20-23] Hence, a fast and efficient re-establishment of a functional and self-tolerant immune system is favorable for successful HSCT outcome. Long-term disease free survival comprises the eradication of the tumor that mainly occurs trough the combination of cytoreductive conditioning and the GVT activity. The histocompatibility differences between donor and host T cells are crucial for a strong and sustained GVT effect.[24,25] Unfortunately, in allo-HSCT, the donor T cells may react against the same epitopes that are also expressed on non-malignant host cells and thus can mediate an immune response against healthy recipient tissues. This immunological reaction results in GVHD which occurs in 40-60% of the patients receiving an allo-HSCT[26,27] and is associated with significant mortality in approximately 15% of transplant recipients.[28-30] Importantly, GVHD increases the likelihood to be diagnosed with an opportunistic infection as it delays reconstitution of naïve T cells. Hence, this TRT is a detrimental factor for immune regeneration. The following chapters will first present current knowledge concerning I) the immunopathophysiology of GVHD and II) the immune regeneration following allo-HSCT and then address the known mechanistic relationship between these two events.[31,32]

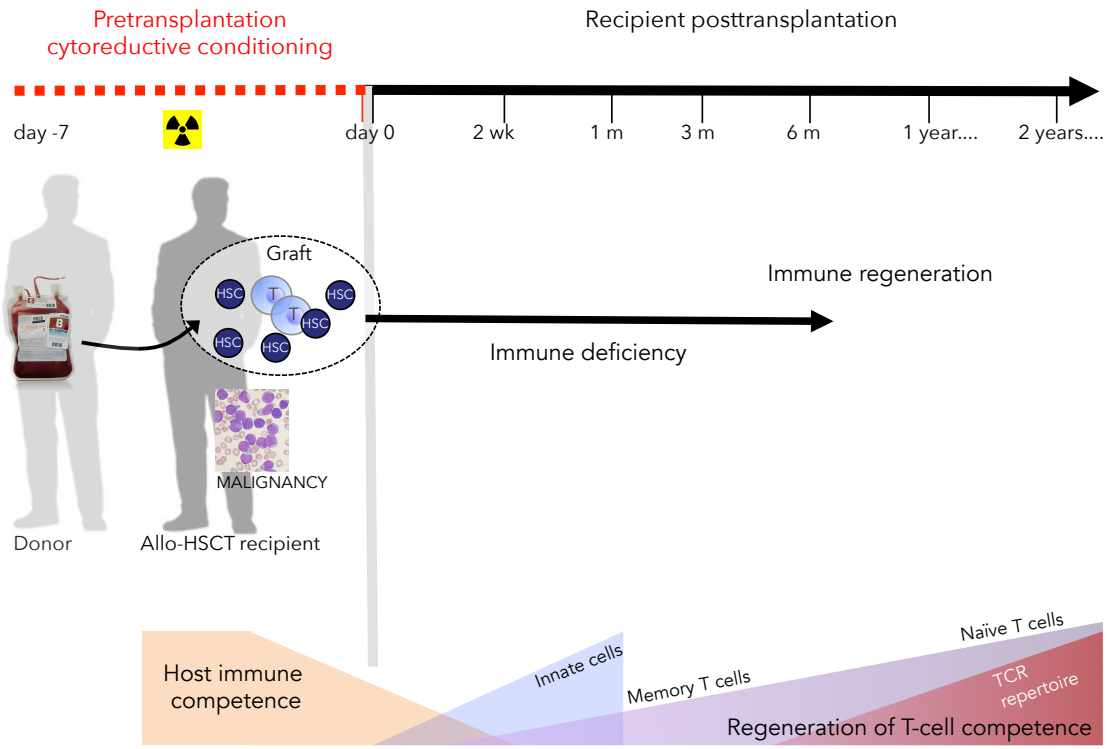


Figure 2: Successful clinical outcome of allo-HSCT. *Prior to allogeneic hematopoietic stem cell transplantation (allo-HSCT), the patients receive pretransplantation cytoreductive conditioning that eradicates the bulk of the tumor. However, this intervention also comprises host immune competence, resulting in an immune deficiency within the first weeks and months after allo-HSCT. This phase of immune deficiency increases the susceptibility to opportunistic infections and residual tumor cell growth. Successful clinical outcome of HSCT is defined trough three main factors: I) Disease-free survival due to the graft-versus tumor (GVT) effect provided by donor mature T cells. II) No complications, as defined by the absence of transplant-related toxicities from graft-vs-host disease (GVHD) and conditioning. III) A fast and efficient establishment of donor HSC-derived immune competence.*

2.1.3 Graft versus host disease (GVHD)

GVHD is the major complication after allo-HSCT whose development and severity directly correlates with the number of transferred donor T cells and degree of major histocompatibility complex (MHC) and minor histocompatibility antigen (miHA) mismatch between the donor and host tissues.[33-37] Billingham described as early as in 1959 three main factors leading to GVHD: I) The host must be immunocompromised II) the host must express tissue antigens that differ to the ones contained in the graft (histo-incompatible) III) the graft must contain competent cells that are able to induce an immune response.[30,38] For long time, GVHD was categorized into its two distinctive forms according to the time when symptoms appear: The symptoms that emerged within the first 100 days after transplantation were characterized as the acute form of GVHD (aGVHD) that is induced by alloreactive donor T cells whereas symptoms that arose 100 days

post-transplantation were categorized as the chronic form (cGVHD), which is mainly characterized by autoimmune manifestations.[65,39] However, recent advances in HSCT suggested to classify GVHD based on the manifestations of GVHD and disease severity rather than time of onset post-HSCT. This new classification was proposed by the National Institutes of Health (NIH) consensus criteria in 2005 and provided new guidelines for global assessment of GVHD severity.[40,41]

aGVHD - pathomechanism

The development of aGVHD results from donor T-cell recognition of genetically disparate recipient tissues. In MHC-mismatched settings, donor T cells react against both the antigen presented by the MHC and the MHC molecule itself.[26] In MHC-matched settings, approximately 40% of the patients also develop aGVHD. This fact is due to the genetic loci encoding miHA, which lie outside of the MHC region.[39,42,43] The induction of aGVHD occurs in three main steps (Figure 3): The first step is the activation of antigen-presenting cells (APC) due to conditioning-associated inflammation and the release of pro-inflammatory cytokines due to the underlying disease, thus leading to initial tissue damage.[44] The damage of the gut leads to release of gut bacteria and the expression of pathogen- associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), lipopolysaccharides (LPS) and other chemokines.[45] This mechanism in turn recruits neutrophils, NK cells, macrophages and other innate cells that secrete more pro-inflammatory cytokines such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), IL-1, IL-2, or IL-6, IL-12, IL-21, IL-22 and IL-23.[46,47] This cytokine storm further activates recipient APC.[34] The second stage is the activation, differentiation and expansion of infused naïve donor T cells. Alloreactive donor T cells recognize foreign MHC molecules or self-MHC molecules complexed with foreign peptide allo-antigen. $CD8^+$ donor T cells respond to MHC-class-I molecules that are expressed on almost all nucleated cells, including non-hematopoietic cells, and hence mediate further tissue damage. $CD4^+$ T cells recognize peptides that are presented by MHC-class-II-molecules mostly on hematopoietic cells and differentiate into T helper 1 (T_H1), T_H2 or T_H17 cells. Several studies showed that a T_H1 response mainly mediates aGVHD whereas cGVHD rather involves a T_H2 response.[48,49] Moreover, $CD4^+$ regulatory T cell (T_{reg}) cell numbers are decreased. These cells normally have a protective role as they have a suppressive activity towards allo- and autoreactive T cells.[50,51] In addition to T-cell receptor (TCR)-mediated signals, the co-stimulatory signal molecules (such as CD28, CD80) play a fundamental role in the induction of aGVHD. The migration of activated donor T cells to tissue via blood and lymphatic system induces the effector phase of the GVH reaction characterized by target tissue destruction predominantly in skin, liver and gastrointestinal tract.[52] The preconditioning regimen-induced tissue injury allows facilitated access to these organs.[44] The

progressive destruction of tissue results in further release of inflammatory cytokines such as IL-1, IFNγ, TNFα, IL-17 and may also induce secretion of perforin/granzyme B, Fas/Fas ligand and TNF-related apoptosis inducing ligand (TRAIL). The effector phase of tissue apoptosis-mediated by pro-inflammatory cytokines and cellular effectors leads to further tissue destruction. This may result in end organ damage.[15,53]

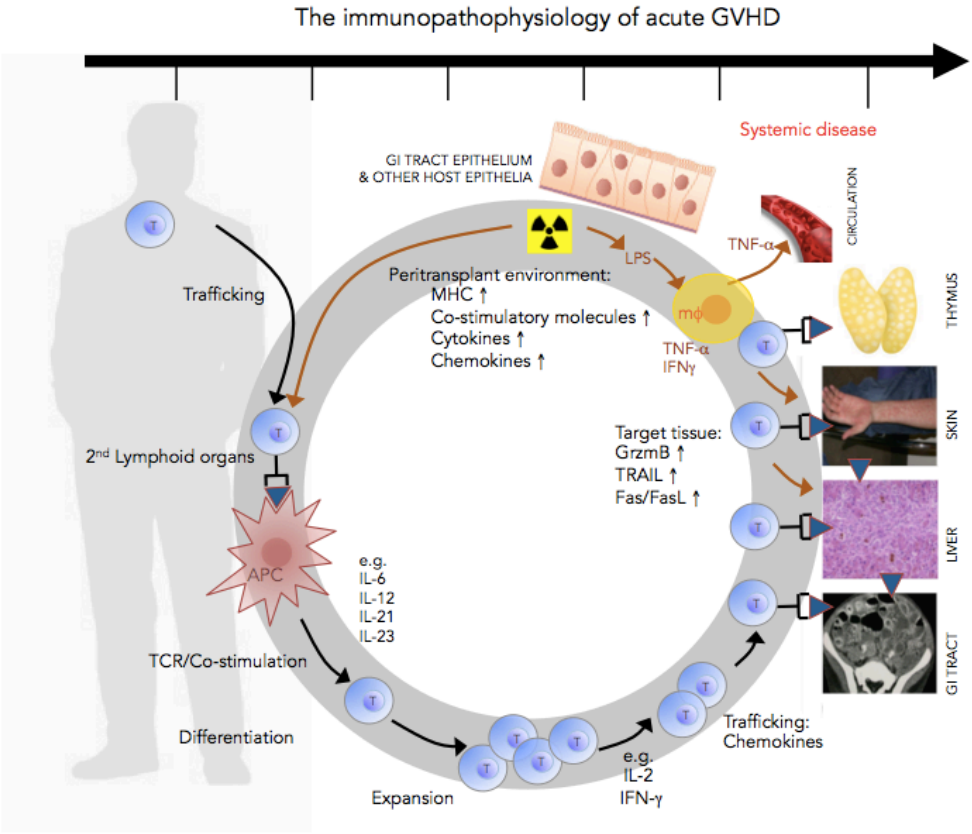


Figure 3: The immunopathophysiology of aGVHD. *Pretransplantation conditioning induces tissue inflammation and cytotoxicity, which results in the secretion of pro-inflammatory cytokines and chemokines and the upregulation of co-stimulatory molecules. In addition, gut destruction releases lipopolysaccharides (LPS), enhancing inflammation and the recruitment of monocytes. This pro-inflammatory environment activates antigen-presenting cells (APC). In parallel, donor mature T cells traffic to secondary lymphoid organs (SLO) where priming occurs after interaction with APCs. Activated donor T cells differentiate and expand, producing more cytokines and further migrate to graft-vs-host disease (GVHD) target tissues, which mainly include the gastro-intestinal (GI) tract, liver, skin and thymus. There, the cytokine storm and cellular effectors mediate further destruction and tissue apoptosis with consequent end organ damage.*

cGVHD – pathomechanism

cGVHD occurs within 3 years post allo-HSCT in 30-60% of patients and resembles autoimmune syndromes characterized by the appearance of scleroderma, bronchiolitis obliterans and fibrosis.[54,55] cGVHD is classified into two categories based on symptoms that might overlap with

typical features of aGVHD.[40] There is a strong association between antecedent aGVHD and autoimmunity in allo-HSCT recipients.[56,57] Correlative human studies and preclinical murine models have advanced several hypotheses and thus far have proposed at least four distinct pathomechanisms to explain cGVHD: I) The damage of the thymus caused by prior aGVHD impairs the process of negative selection to eliminate T cells responsible for autoimmunity.[58] II) Aberrant activation of allo- and autoreactive T- and B cells favors the production of auto-Ab.[59] The pathogenic role of B cells during cGVHD is supported by preclinical studies showing that cutaneous sclerosis could be improved in patients with cGVHD by the depletion of $CD20^+$ B cells with an α-CD20mAb therapy (Rituximab).[60] Moreover, increased levels of B-cell activating factor (BAFF) were reported to promote survival of allo- and autoreactive B-cells.[61,62] III) Fibrogenic cytokines such as IL-10 or transforming growth factor-β (TGFβ) released by macrophages also play an important role in the pathophysiology as they were shown to ameliorate murine cGVHD after neutralization.[63] IV) Platelet-derived growth factor (pDGF) receptors on fibroblasts bind auto-Ab leading to further cytotoxicity, chronic inflammation and fibrosis.[64,65] All four pathomechanisms need to be controlled in order to mitigate cGVHD after allo-HSCT. Therefore, many ongoing investigations focus on the role of T_{reg} cells and cGVHD.[66]

Current GVHD prophylaxis and therapy

The standard prophylactic therapy for GVHD are calcineurin inhibitors (CNI) that dampen T-cell activation.[67] The most widely used CNI are cyclosporine A and tacrolimus. Methotrexate (MTX) is a cytotoxic drug that exerts anti-inflammatory effects by attenuating T-cell activation when given at low doses. Several studies suggested a combination therapy of CNI and MTX with improved outcome in comparison to CNI alone.[68] Currently, this combination is the most commonly used therapy in patients receiving allo-HSCT from URD. Another first-line standard therapy is the application of corticosteroids[69] but almost 50% of the patients will not respond and develop steroid-refractory GVHD.[70] Unfortunately, the administration of general immunosuppressants further exacerbates posttransplantation immune deficiency and predisposes patients to opportunistic infections. A more selective targeting includes the depletion of T cells from the BM (TCDBM, T-cell depleted bone marrow) before allo-HSCT. Another possibility to deplete T cells is by using anti-thymocyte globulin as part of prophylaxis following allo-HCST.[71] The administration of cyclophosphamide shortly post-HSCT selectively kills fast proliferating cells which mainly include alloreactive donor T cells after priming without affecting T_{regs}.[72,73] Thus, the depletion of T cells remains an effective way to prevent GVHD. Unfortunately, this approach decreases GVT activity and has also a negative effect on posttransplantation immune regeneration (see below).[74,75] Although the use of standard interventions either prophylactically or

therapeutically potently diminish donor T-cell expansion and differentiation, the fact that high number of patients still develop GVHD highlights the need for better intervention strategies. Preventing GVHD while retaining the beneficial GVT effect and avoiding opportunistic infections presents a major challenge for successful HSCT. Hence, a deeper understanding of cellular and molecular key mechanism that cause GVHD are crucial in order to find new intervention strategies to prevent or control the disease.

2.2 T-cell development post allo-HSCT

As stated in the previous chapter, successful allo-HSCT critically depends on a full reconstitution of immune competence following conditioning. Preclinical and clinical studies have examined the kinetics of T-cell recovery following allo-HSCT.[76-78] This process normally operates along two different pathways that occur in parallel as illustrated in Figure 4. The thymus-independent pathway involves peripheral T cells, including either remaining host T cells that had survived pretransplantation conditioning or newly infused donor mature T cells that expand in the periphery. The thymus-dependent pathway in contrast involves donor-HSC derived T cells that are generated *de novo* in the recipients' thymus and provide an efficient and long-lasting T-cell immune competence.

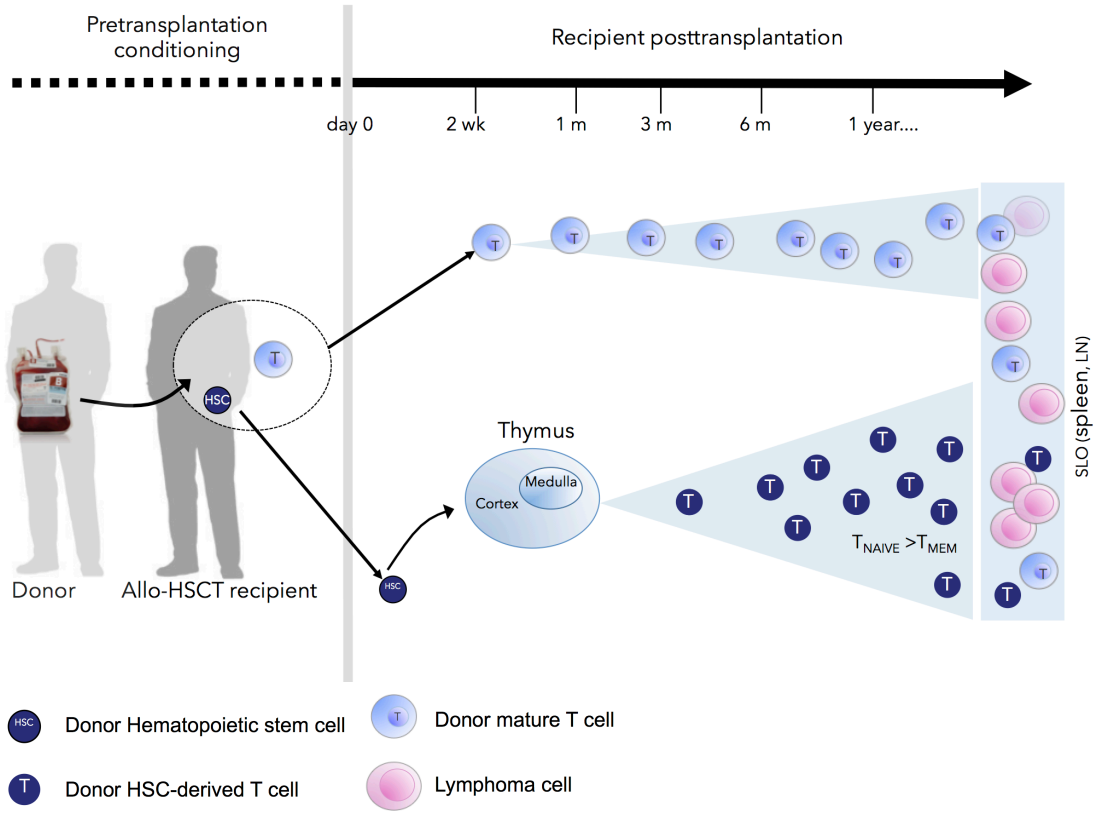


Figure 4: Scheme of T-cell regeneration pathways after allo-HSCT*. The graft contains hematopoietic stem cells (HSC) and donor mature T cells, resulting in two different pathways of T-cell recovery that occur in parallel. In the thymus-dependent pathway, HSC-derived T-cell progenitors migrate from the peripheral blood (PB) to the thymus, where they undergo normal positive and negative selection and are released to the periphery as naïve T cells. The thymus-independent pathway is based on the peripheral expansion of host T cells and donor mature T cells that directly migrate to SLO, where they encounter antigens from pathogens and tumor cells. This figure is adapted from Krenger et al, Blood 2011.*[21]

2.2.1 Thymus-independent T-cell development

The initial wave of T-cell recovery shortly after allo-HSCT involves the peripheral expansion of pre-existing host T cells and of donor mature T cells that are contained in the graft.[79] In the first period post allo-HSCT donor T cells serve as a sufficient protection against infections since they have a mature phenotype and directly migrate to secondary lymphoid organs (SLO) where they proliferate upon exposure to nominal antigens including those which were previously encountered.[21,80] Indeed an important clinical observation is the fact that the transfer of TCDBM is strongly associated with Cytomegalovirus (CMV) infections and Epstein-Barr Virus-associated disorders.[81,82] However, expanding donor T cells have a limited and skewed TCR repertoire, which is not sufficient for a comprehensive response to a wide diversity of foreign antigens. The diversity of the T-cell pool largely depends on the diversity of the mature T cells that served as the source for expansion. Moreover, the clonal expansion is only transient and characterized by clonal exhaustion and activation-induced cell death of alloreactive donor T cells.[78] Although $CD8^+$ T cells can recover rapidly by peripheral homeostatic expansion, the same mechanism is not enough to replenish the $CD4^+$ T cells. Thus, there is much evidence that the thymus-independent T-cell regeneration is not able to reconstitute the complete host immune system and relies on the thymic output.

2.2.2 Thymus-dependent T-cell regeneration

Sustained immunity with a high TCR repertoire can only be achieved by a sizeable pool of naïve T cells whose formation relies on the *de novo* generation of the host thymus post allo-HSCT. The thymus-dependent pathway involves donor-BM derived thymic progenitors that are contained in the graft and replenish the thymus to undergo normal T-cell development. In the absence of TRT, donor-BM derived precursors undergo normal developmental processes including selection mechanism that are essential to eliminate autoreactive T cells, as outlined in the following chapters.[78,80]

2.2.2.1 Normal thymic T-cell development

The thymus is a primary lymphoid organ that is responsible for the maturation of T cells and comprises a subcapsular region, the cortex, cortico-medullary junction and medulla.[83] The thymic microenvironment is a three-dimensional network consisting of stromal cells such as cortical and medullary thymic epithelial cells (cTEC and mTEC respectively), non-epithelial hematological cells such as dendritic cells (DC) and macrophages and fibroblasts that together build a complex scaffold. These cell types are responsible for T-cell survival, expansion, differentiation and central tolerance.[84,85,86] The access of T-cell progenitors to the thymus depends on the expression

of selectins such as P-selectin, integrins and chemokine receptors like C-C chemokine receptor 7 (CCR7).[87-90] HSC-derived T-cell precursors enter the thymus as early thymic progenitors (ETPs) expressing CD117 (c-Kit) Sca-1 and CD44 whereas they are negative for the lineage markers (Lin) CD3, CD4, CD8, CD11b, CD11c, CD19 and CD25.[91,92] After entry, ETPs migrate through the cortex as a double negative (DN) subset for CD4 and CD8 surface molecules. These DN stages can further be divided in DN1-DN4 (DN1: $CD44^{+}CD25^{-}$, DN2: $CD44^{+}CD25^{+}$, DN3: $CD44^{-}CD25^{+}$, DN4: $CD44^{-}CD25^{-}$).[93] Within the DN1 stage, the cell population has still multi-lineage potential for lymphoid and myeloid cells such as B cells, T cells, natural killer cells (NK cells) or DCs. With the additional expression of CD25 in the DN2 stage the cells lack B cell potential but keep their potential to become NK cells, DC or T cells. In the DN3 stage, the TCRβ chain rearrangement occurs together with the expression of the CD3 molecule.[94] In this stage the T-cell lineage commitment is completed. With their productive TCRβ rearrangement, the thymocytes give rise to the CD4 and CD8 double positive (DP) subset that comprises around 85% of all cells in the thymus.[95]

Positive selection

Once thymocytes terminate the first wave of proliferation, the α-chain locus begins to rearrange. As αβ-DP cells, they undergo a process of positive selection that shapes the TCR repertoire. This process takes place in the cortex, which contains cTEC that are identified in the murine system through markers such as intracellular cytokeratin (CK) 8 and CK18 as well as the epithelial cell adhesion molecule (EpCAM; CD326) and the surface molecule Ly51.[96] Together with other stromal cells, cTEC provide soluble factors such as interleukin 7 (IL-7), Fms-like tyrosine kinase 3 ligand (Flt3L), C-C chemokine ligand 25 (CCL25) and membrane-bound proteins, including MHC, that are crucial for survival, division and migration of thymocytes.[86,97,98] The positive selection process controls whether thymocytes can recognize self-peptide:self-MHC complexes. If the cells fail to recognize MHC molecules they will die by neglect. Only about 3-5% of all thymocytes will survive and further develop. Positively selected DP cells downregulate CD24 and CCR7 and further differentiate into CD4 or CD8 single positive (SP) cells that are restricted to recognize MHC II or MHC I, respectively. As CD4 or CD8SP thymocytes, they traffic to the medulla.

Negative selection

Self-tolerance of the nascent TCR is attained through negative selection in the thymic medulla (Figure 5). The medulla contains mTEC that are identified in the mouse and in human through CK5, CK14, EpCAM and *Ulex europaeus* agglutinin-1 (UEA-1).[96,99,100] Mature mTEC express CD80 and high levels of MHC II ($mTEC^{high}$) and are the major contributors of negative selection.

The exposure of self-antigen, including those with highly restricted tissue expression is essential for clonal deletion. Under physiological conditions, interactions of immature T cells and a self-peptide with too high affinity/avidity will mediate an intracellular signaling pathway that induces programmed cell death in developing thymocytes (Figure 5). In order to become negatively selected and eliminated, autoreactive T cells upregulate Helios and programmed cell death protein 1 (PD-1).[101,102]

Thymic ectopic expression of many tissue-restricted peripheral self-antigens (TRA) that can be presented to developing thymocytes is a distinct property of $mTEC^{high}$.[103] This so-called promiscuous gene expression (pGE) is a stochastic process in which only a limited number of mTEC (1-3%) express a given TRA.[104,105,106] TRA expression is partly controlled by the TF *autoimmune regulator* (*Aire*).[107,108] Importantly, intimate associations exist between perturbations in TRA expression and the susceptibility to autoimmunity in both animals and human.[109-111] The importance of *Aire* is illustrated in patients that have a mutation in *Aire.* This defect causes autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED; alternatively autoimmune polyglandular syndrome type I APS-1).[112] Similarly, Aire deficient mice ($Aire^{-/-}$) have an impaired negative selection.[113] Consequently, loss of TRA expression by lack of $Aire^{+}mTEC^{high}$ cells results in *de novo* generation of autoreactive T cells.[114] Because negative selection is not absolutely efficient, an alternative to clonal deletion is the development of $CD4^{+}CD25^{+}$ Forkhead-Box-Protein $P3^{+}$ (FoxP3) thymocytes that are released to the periphery as tolerogenic thymic T_{regs}.[115] Only 1-2% of all T cells survive these selection processes and are allowed to leave into the periphery.[116]

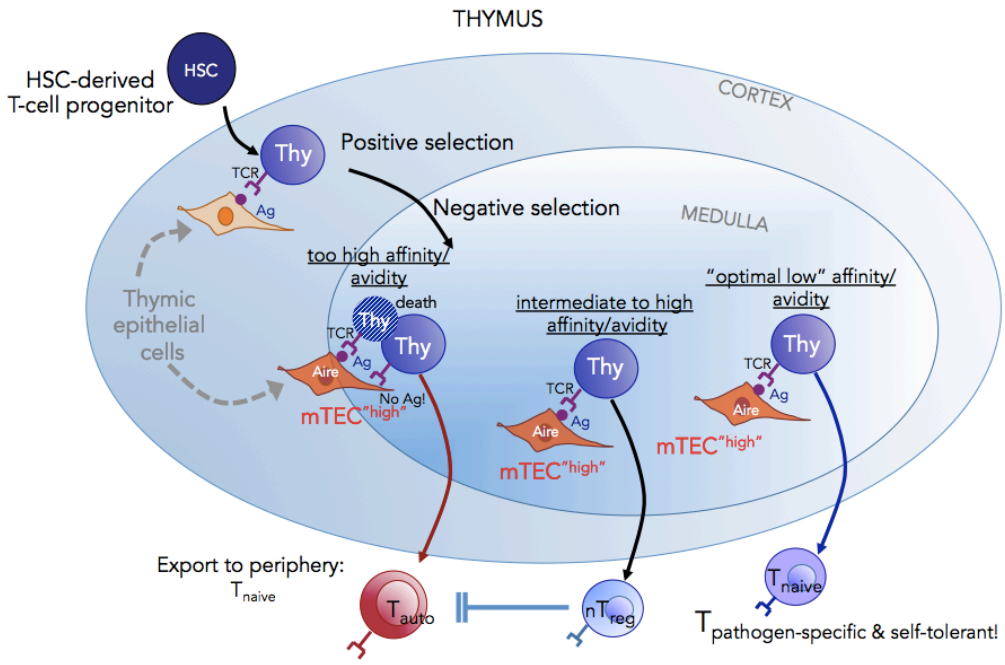


Figure 5: Positive and negative selection in the thymus. *HSC-derived T-cell progenitors enter the thymus and migrate to the cortex, where thymocytes (Thy) undergo positive selection. A self-peptide:self-MHC complex is presented by cortical thymic epithelial cells (cTEC) to immature thymocytes. Thymocytes that do not respond die by neglect. Positively selected thymocytes migrate further to the medulla where they undergo negative selection. Here, autoimmune regulator expressing medullary TEC ($Aire^{+}mTEC^{high}$) present in conjunction with dendritic cells (DC) many tissue restricted antigens on their surfaces. If thymocytes respond with too high affinity/avidity, they are negatively selected and die due to programmed cell death. Thymocytes that respond with intermediate to high affinity/avidity differentiate into T_{regs}. Thymocytes that respond with an optimal low affinity/avidity survive negative selection and are released into the periphery as naïve T cells.*

After complete maturation, thymocytes traffic from the thymus into the periphery via the bloodstream. The emigration step is associated with the downregulation of the surface marker CD69 and the presence of the lipid molecule sphingosine-1-phosphate (S1P) in the blood.[117] In order to exit the thymus, T cells express the G-protein coupled receptor (GPCR) S1P receptor 1 ($S1PR_1$) on their surface (see chapter 2.3).[118,119]

2.2.3 The effect of thymic dysfunction on immune reconstitution after allo-HSCT

Newly generated T cells have a naïve phenotype, a broad TCR repertoire and are specific to foreign pathogens. Importantly, these cells are not host-reactive following allo-HSCT due to their thymic education. This *de novo* production of thymus-derived T cells is, however, a long process and 1-2 years are required to reach a normally sized T-cell pool.[120,31] The potential for thymic renewal is present in younger patients but limited or even absent in older patients (>50 years of

age).[121] Nevertheless, the phenomenon of "thymic rebound" can be observed in elderly patients, where the thymus enlarges after engraftment within the first weeks following HSCT.[21,80] The numbers of exported naïve T cells to the periphery post allo-HSCT can be predicted by the kinetics as well as extent of thymopoietic reconstitution and is detected in the periphery by the emergence of $CD45RA^+$, $CD62L^+$ cells.[76,78] The quality of newly generated T cells can be assessed by the determination of TCR diversity and function. The TCR repertoire can be measured by flow cytometry or polymerase chain reaction (PCR), where the complementary determining region 3 (CDR3) is typed or levels of TCR rearrangement DNA excision circles (TREC) are determined.[122,123] A quantitative analysis of the function can be made by *ex vivo* stimulation of T cells and their capacity to secrete cytokines that are measured by Elispot.

An efficient T-cell regeneration depends on a functional thymus but the latter can be affected by several risk factors such as disease status, age of the patient, the kind of preconditioning and the presence of GVHD.[21,79,124] TRT stemming from cytoreductive regimens and GVHD harm the thymus and cause a poor clinical outcome post-HSCT characterized by immune deficiency and possibly autoimmunity. The presence of aGVHD in allo-HSCT recipients represents a major predictor for an increased risk for opportunistic infections and GVHD severity is inversely correlated with the ability to generate naïve T cells and thus recover immune competence.[21] This situation is even worsened by the administration of immunosuppressive agents to prevent or treat GVHD. [15] In 1978, Seemayer *et al.* described for the first time the injury of the thymus during aGVHD in murine models.[125] Typical histological features of thymic GVHD include the elimination of TEC, changes in number and composition of thymic subpopulations, phagocytosis of cellular debris and the depletion of Hassall's bodies.[126-128] The impact of alloreactive donor T cells themselves in the context of thymic damage is the subject of intensive investigation. Here, unconditioned mouse models serve as a valuable tool to study the direct influence of donor mature alloreactive T cells on thymic damage. A good mouse model to study is the transplantation of allogeneic splenocytes in a MHC mismatched transplantation setting independent of thymic injury induced by cytoreductive conditioning (parental C57Bl/6 ($H\text{-}2^b$) into the F1 generation (BDF1, $H\text{-}2^{bd}$)).[129] Donor mature T cells infiltrate the thymus shortly after transplantation and react against the recipient's antigens, thus triggering a local inflammatory cascade that induces thymic tissue injury[130] leading to a smaller size and loss of cellularity.[31] It was shown in several mouse models that loss of cellularity is mainly due to the decrease of $CD4^+CD8^+$ cells. This massive reduction of DP cells is largely caused by an impaired cellular proliferation capacity of immature host thymocytes within the DN3 stage, resulting in an accumulation of the DN1 stage and a block in the further development to DP cells.[130] Another reason for DP reduction is increased apoptosis.[77,131] Moreover, in patients with aGVHD, thymic

dysfunction is characterized by a decrease in Dβ-JβTREC and signal joint TREC (sjTREC) frequencies, which results in a normal sj/DβJβ ratio in the peripheral T cells.[132] These observations suggest for impairment prior to CD3,CD4,CD8-triple negative (TN) proliferation as well as later when both TCR chain rearrangements have already occurred.[31,76,133] Importantly, these data indicate that aGVHD impairs thymic function independent of advanced age since the thymus of older individuals displays a decline in the sj/β signature as a result of reduced TN proliferation.[21]

Our lab has previously shown that naïve alloreactive donor T cells can be directly primed by TEC. Upon allorecognition, donor T cells secrete IFNγ, which in turn activates signal transducer and activator of transcription 1 (STAT-1), that stimulates programmed cell death in TEC. A subsequent diminished TEC compartment cannot deliver crucial survival, differentiation and expansion factors for developing thymocytes anymore. Hence, reduction in TEC numbers is a principal injury that limits T-cell maturation and thymic output of naïve T cells following allo-HSCT.[127,129,130] Recently, it has been reported that TRA-expressing $Aire^{+}mTEC^{high}$ are important targets of donor T cells, leading to a declined $Aire^{+}mTEC^{high}$ pool and restricted TRA diversity.[134] Hence, experimental-induced aGVHD weakens the platform for normal thymic repertoire selection, including the selection of conventional and T_{reg} cells. This process likely indicates an association between the alloreactivity in aGVHD and autoimmune syndromes during cGVHD (see chapter below).

2.2.4 Transition from aGVHD to cGVHD

Flowers and colleagues suggested that the mechanisms which are involved in the respective pathologies of aGVHD and cGVHD are not the same and that cGVHD is not simply the end stage of aGVHD.[135] Several experimental transplantation models now provide evidence that the decrease in T_{regs} and a damaged thymic microenvironment during aGVHD may be crucial events that lead to the chronic form of GVHD.[55,136-138] During aGVHD, alloreactive T cells attack TRA-expressing $mTEC^{high}$ in the thymus that are crucial for negative selection. Importantly, many repressed TRAs are specific for tissues known to be major targets of (human) cGVHD (i.e. skin, eye).[134] The restricted TRA diversity expression on $mTEC^{high}$ hence suggests for an extrathymic presence of autoreactive T cells ("Hit-1"). Together with B cell dysregulation and T_{reg} deficiency ("Hit-2") this may lead to the development of cGVHD.[139] Based on this "2-hit model",[139] an etiological link between autoimmunity and antecedent alloreactivity was proposed (Figure 6).[138]

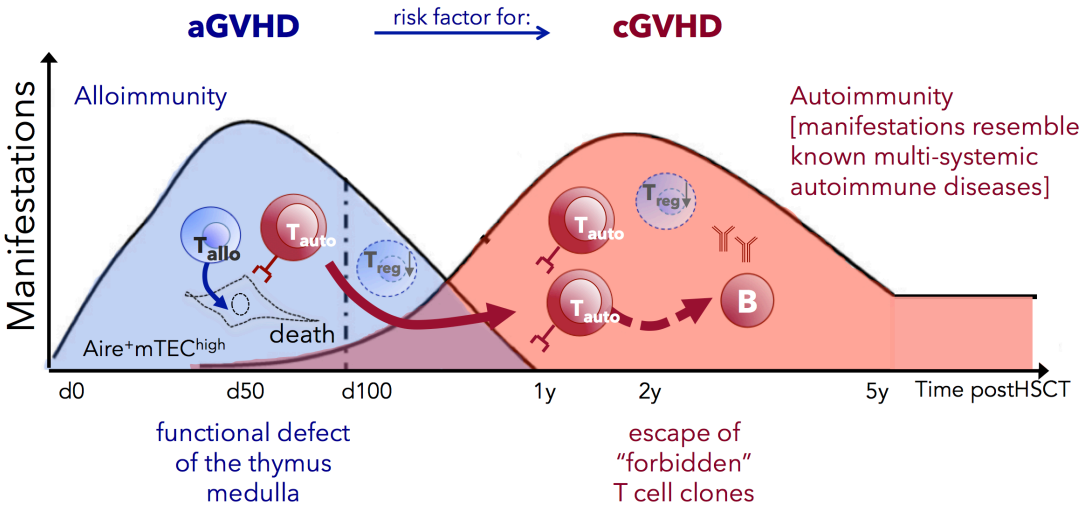


Figure 6: Suggested mechanism for the transition from aGVHD to cGVHD. *During acute graft vs-host disease (aGVHD), alloreactive donor mature T cells infiltrate the thymus and damage tissue-restricted antigen-expressing Aire^{+}mTEC^{high} that are crucial for negative selection. This mechanism leads to an impaired negative selection process resulting in a decreased number of T_{reg} and probably the production and subsequent release of de novo generated autoreactive T cells into the periphery. There, autoreactive T cells might induce autoimmune symptoms that are manifested in the chronic form of GVHD. This figure is adapted from Pavletic et al., Hematology 2012*[140] *and data from Flowers, Rangarajan, Parkman*[135,139,141] *and Krenger were added.*

2.2.5 How may post-HSCT thymic function be improved?

The immunological complexity post allo-HSCT shows that an impaired T-cell reconstitution due to thymic dysfunction during aGVHD has severe consequences for transplant outcome. A poor T-cell output not only diminishes the protection against infections, it also increases the risks for relapse. Moreover, the diminished HSC-derived *de novo* T-cell development results in a shrinking peripheral naïve T-cell pool, skewed TCR repertoire, diminished T_{reg} numbers and possible production of autoreactive T cells and the development of cGVHD.[139]

To restore long-term T-cell immunity post-HSCT faster and more efficiently, approaches that enhance thymic function would offer an attractive perspective. Several therapeutic und prophylactic strategies have been investigated to avoid detrimental effects on the *de novo* generation of T cells post allo-HSCT. For example, an efficient way to enhance thymic function is the administration of fibroblast growth factor (Fgf)-7 (also known as keratinocyte growth factor, KGF or Palifermin). Fgf-7 is normally expressed by stromal cells and T cells within the thymus and stimulates thymic T-lymphopoiesis. Postnatal TEC require external growth factors for proliferation as it was demonstrated that immature as wells as adult mTEC and cTEC still

express the Fgf receptor FgfR2IIIb. The prophylactic administration of recombinant Fgf-7 indeed enhanced thymic function and (re-)generation of the thymic stromal compartment post-HSCT regardless of conditioning and aGVHD in mice by protecting TEC from cytotoxicity.[142,143] As a result of an restored TEC compartment, a functional T-cell development was maintained and subsequent enhanced T-cell output positively affected the peripheral T-cell pool.[76] Unfortunately, the relevance of Palifermin is uncertain since data from clinical trials showed that Palifermin has not ameliorated GVHD and not improved T-cell regeneration, and was also particularly ineffective in preventing signs of autoimmunity. Moreover, the standard administration of steroids in aGVHD negatively regulates Fgf7 mRNA.[144]

Alternative strategies to enhance thymic formation and maintenance in the transplant setting, and which are currently tested in clinical trials, include hormonal modulation (i.e. growth hormone and insulin-like growth factor 1 administration) and cytokines (IL-7 and IL-22).[79] Preclinical studies have shown that exogenous IL-7 administration indeed enhances thymopoiesis and boosts T-cell recovery in murine syngeneic but also allo-HSCT.[145-147] In patients, recombinant human IL-7 was reported to increase T-cell numbers and patients receiving human IL-7 administration also showed a broader TCRβ repertoire in comparison to untreated patients.[148] The cytokine IL-22 also plays an important role in thymic recovery as intrathymic ILCs produce IL-22 as a response to acute thymic damage. IL-22 directly promotes proliferation and survival of TEC and supports the microenvironment in thymocyte renewal.[149] Human recombinant IL-22 is currently tested in a clinical trial in aGVHD patients (#NCT02406651).

These approaches need further testing but it is also clear that new and improved strategies are additionally needed that protect or maintain the TEC compartment and thymic microenvironment despite TRT to enhance T-cell recovery and a functional peripheral T-cell pool post allo-HSCT.[78] Instead of the initial focus on boosting thymic recovery after damage, an alternative approach would be to prevent thymic injury before it occurs. One possibility to prevent thymic damage could be by interfering with alloreactive donor T-cell migration to the thymus, as further elaborated in the next chapter.

2.3 Sphingosine 1-Phosphate (S1P) and the S1P Receptors (S1PR)

S1P is a lysophospholipid that induces many functional responses and actions that are important for cell growth, survival, differentiation, lymphocyte traffic, vascular integrity and cytokine/chemokine production.[150,151] The precursor of S1P, sphingomyelin, is normally integrated in the plasma membrane. In response to cytokines, it converts to the sphingolipid ceramide and then becomes phosphorylated either in the cytoplasm by the sphingosine-kinase 1 (SPHK1) or in the endoplasmatic reticulum, mitochondria or nucleus by the SPHK2.[152] In the ER, the phosphorylated sphingosine is irreversibly degraded or dephosphorylated.[119] In the mitochondria or nucleus, S1P has direct intracellular targets such as NF-κB signaling via TNF receptor-associated factor 2 (TRAF2).[153] In the cytoplasm, the phosphorylated sphingosine is transported to the blood stream via a S1P transporter. High levels of S1P in the blood plasma are produced by erythrocytes[154] whereas in the lymph, endothelial cells mainly provide S1P.[155] S1P binds with high-affinity a family of GPCRs to induce further signaling pathways in an autocrine or paracrine manner.[118,119] The S1P-receptor (S1PR) family consist of 5 different receptors; $S1PR_{1\text{-}5}$.[156] $S1PR_1$ was the first described receptor[157,158] and was shown to be highly involved during embryogenesis in the formation of the vascular network.[159,160] Relevant to the present thesis is the fact that also lymphocytes, mostly B- and T cell express the $S1PR_1$. The persistent expression of $S1PR_1$ allows T cells to migrate and re-cycle within the body.[161] The migration process is determined by the concentration of S1P itself, suggesting for a tightly regulated gradient between S1P levels in the PB, in the lymph and within SLO in a concentration-dependent manner.[119,162]

2.3.1 S1P-$S1PR_1$ axis in T-cell development and homeostasis

The importance of $S1PR_1$ in lymphocyte egress from the thymus to the periphery was shown in a $S1PR_1$-deficient mouse model in the study of Matloubian and Allende *et al.*.[163,164] Thymii of $S1PR_1$-deficient mice showed an accumulation of mature CD4/CD8SP thymocytes and a decreased proportion of less mature DP cells. Importantly, these DP cells had no phenotypical changes typical for their maturation status, suggesting for a normal thymic development but impaired egress from the thymus.[163,164] During maturation of DP to CD4/CD8SP, $S1PR_1$ mRNA is upregulated in thymocytes by the TF Krüppel-like factor 2.[165] These and other studies indicated that $S1PR_1$ is a key regulator for the egress of T cells from the thymus in response to surrounding S1P that is produced by neural crest-derived perivascular cells.[163,166] As soon as T cells emigrate from the thymus and enter the circulation, they internalize the receptor in response to high S1P concentrations in the blood or lymph. This internalization allows T cells to further traffic to SLO

where they survey the organ for foreign antigens. In a non-inflamed lymph node, S1P levels are lower than in the blood and mature T cells re-express $S1PR_1$. If the lymphoid tissue is inflamed, T cells get activated and upregulate CD69 on their surface. This expression induces internalization and degradation of $S1PR_1$ to prolong the presence of T cells at the site of inflammation.[167] After several cell divisions, newly generated effector T cells can then upregulate $S1PR_1$ again to leave the peripheral tissue and enter the circulation.[119,168] In $S1PR_1$-deficient mice this egress from SLO is blocked.[168,169]

2.3.2 Pharmacological S1PR(1) modulation

The enhanced understanding of lymphocyte egress and traffic paved the way to use the $S1P/S1PR_1$ axis for therapeutic interventions in situations where unwanted T-cell responses play a role in disease pathogenesis. Thus, manipulating S1P or its receptor might lead to lymphocyte trapping within SLO where T cells may maintain their immune response, while the migration to possible target organs is prevented. This idea makes it very attractive to use this axis as a target of immunosuppressive drugs. In 1996 the compound FTY720 (Fingolimod) was described, which acts as S1PR agonist. FTY720 was then used in many preclinical investigations where, for instance, it was shown to induce long-term graft acceptance in rats and dogs.[170-174] Since then a large number of clinical trials have been performed which could clearly demonstrate the efficacy of FTY720 in unwanted T cell responses in solid organ transplantation settings, autoimmune diseases and cancer due to its agonistic effect on $S1PR_{1,3-5}$.[172,175,176]

2.3.3 Role of $S1PR_1$ agonism in the context of GVHD, engraftment and GVT activity

Engraftment

HSC-derived engraftment post allo-HSCT remains a crucial factor for a successful HSCT outcome. The observation that CXC-chemokine receptor 4 (CXCR4), an important regulator in BM-derived cell engraftment from and to the BM cavity, is inhibited by S1P under physiological conditions, indicated the dependence of CXCR4 signaling on $S1PR_1$ agonism.[177,178] Indeed, FTY720 was shown to enhance BM-derived allo-reconstitution via the increased expression of CXCR4 on HSC-derived progenitors resulting in an improved BM-cell engraftment. However, these and other studies showed that improved allo-engraftment was only true for the homing of HSC to the BM and by the fact that HSC were retained in the niches.[179,180]

GVHD

During aGVHD, activated APCs can prime donor T cells within SLO. These alloreactive T cells then migrate to GVHD target organs including skin, liver, gut and thymus, shortly after allo-HSCT and induce tissue damage[39] (as shown before, T cells may also be activated directly in the thymus). Thus, T-cell migration from SLO to target tissues is crucial for the GVHD effector phase and highlights the importance of interfering with T-cell trafficking. With its agonistic effect on $S1PR_1$, FTY720 was shown to efficiently reduce alloreactive donor T-cell export from SLO and hence to diminish GVHD induction in target organs including the migration to the thymus.[181,182] Moreover, FTY720 was shown to promote $FoxP3^+T_{reg}$ cell differentiation from thymic T_{reg} precursors and function of mature T_{reg} via the functional antagonism of $S1PR_1$, which normally activates mTORC1 signaling that in turn activates Smad3 and hence antagonizes T_{reg} differentiation. [183] Thus, the promotion of higher incidence of T_{reg} differentiation and function of mature T_{reg} by FTY720 additionally supports the potential of $S1PR_1$ agonism in GVHD prevention. The agonistic binding to $S1PR_1$ by FTY720 leads to a phosphorylation of FTY720 and a subsequent internalization of the receptor.[184-186] Although the receptor is internalized, the binding of pFTY720 to $S1PR_1$ leads to a permanent signaling.[187] Thus, T cells stay resistant to egress-signals coming from S1P and persist within SLO.[171]

GVT

S1PR modulation via FTY720 was shown to efficiently separate GVHD from the beneficial GVT effect in preclinical models.[182,188] The direct effect of S1P on tumor cells was described in several studies involving overexpression, inhibition or knockdown of the SPHK1 activity that normally promotes cell growth and inhibits apoptosis. An upregulation of SPHK1 for instance increases the production of S1P, leading to more activation and induced inflammation and directly correlates with poor cancer prognosis, suggesting for SPHK1 inhibitors as cancer therapy.[189,190] Also, the S1PR agonist FTY720 was reported to have anti-cancer properties in leukemia and lymphoma by inhibiting cell growth and inducing apoptosis.[191,192] Another potential reason for tumor eradication in GVHD during FTY720 administration could be the fact that cytotoxic NK cells are not limited in their migration by FTY720 and can migrate to the side of the tumor to support tumor killing.[170,178]

2.3.4 The $S1PR_1$-specific agonist KRP203

The molecular basis underlying the mode of action of FTY720 has only recently been investigated. The advantage of FTY720 over other immunosuppressants is the fact that it does not dampen B- or T-cell function but interferes with T-cell trafficking by trapping lymphocytes within SLO. In the context of GVHD, this further provides the GVT effect while the migration of alloreactive T cells to target tissue can be reduced. However, the undesirable risk of cardiac diseases due to $S1PR_3$ binding overcame the beneficial effect of lymphocyte trapping upon FTY720 administration.[193,194] These findings suggested using more selective S1PR agonistic compounds with an exclusive binding of $S1PR_1$ (Figure 7). The novel compound 2-amino-2-propanediol hydrochloride (KRP203) is the next generation of a more specific $S1PR_1$ modulator.[195] In addition, KRP203 has already been shown to induce permanent acceptance of pancreatic islet allografts and an even enhanced T_{reg} frequency and function in a mouse model.[196] Based on the beneficial function of T_{regs} and the highly selective affinity to $S1PR_1$, KRP203 may be a promising novel approach for GVHD prevention. KRP203 is currently tested in a clinical trial phase I at the University Hospital of Basel for GVHD prevention in patients undergoing allo-HSCT (#NCT01830010). The estimated primary completion of the study is expected in August 2018.[197]

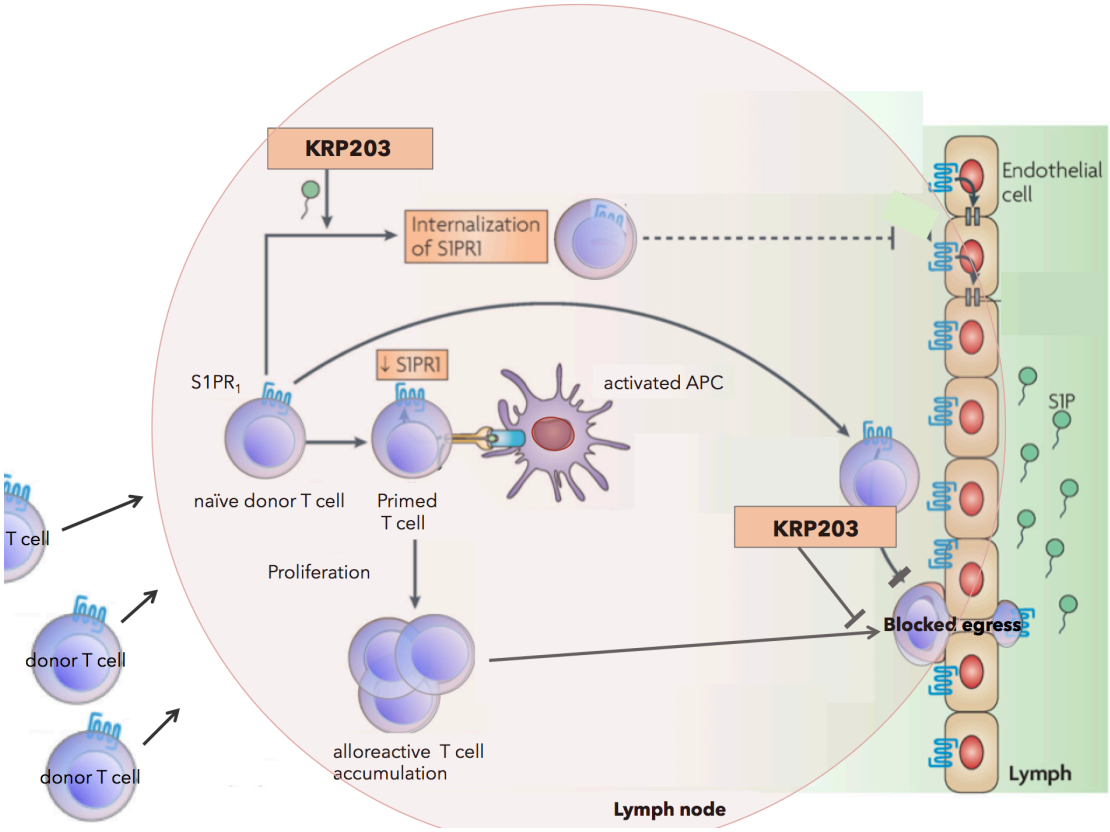


Figure 7: Suggested mode of action of KRP203 during aGVHD. *Sphingosine-1-phosphate receptor 1 (S1PR$_1$) is mainly expressed by naïve T-lymphocytes in order to egress out of secondary lymphoid organs (SLO) by chemoattractance by S1P in the blood. In acute graft-vs-host disease (aGVHD), the S1PR$_1$ agonist KRP203 blocks the export of donor mature T cells once they migrated to SLO so they cannot migrate to any aGVHD target organ while maintaining graft-vs-tumor (GVT) activity at these sides. This figure is adapted from Rivera et al., Nature Immunology Reviews 2008.*[198]

3. Aim of the Project and Hypotheses

Before initiation of my PhD thesis, it was known that I) acute thymic GVHD is induced by alloreactive donor T cells that have entered the thymus and II) that thymic injury is characterized by a diminished pool of $Aire^{+}mTEC^{high}$ and consequently a loss of thymic ectopic expression of tissue-restricted self antigens (TRA).[134] These observations raised the question whether functional impairment of the thymic medulla may be responsible for a defect in thymic self-tolerance induction and subsequently the release of autoreactive T cells into the periphery in the course of disease. Since aGVHD predisposes to cGVHD with autoimmune manifestations, it seemed possible that loss of central tolerance induction provided a pathogenic link between autoimmunity and antecedent alloreactivity. As a corollary, the interference with donor T-cell migration should prevent thymic injury and hence allow for a normal posttransplantation T-cell development in the absence of autoreactive T-cell generation. Founded on these hypotheses, I intended to investigate as core of my PhD thesis the following two interrelated specific aims:

Specific aim 1: To test whether mTEC injury and consequent impairment of thymic TRA expression licenses the de novo generation of autoreactive T cells in aGVHD

To address this hypothesis the following questions were asked:

1. *Is there direct evidence that de novo production of TRA-specific T cells during aGVHD is a consequence of impaired ectopic TRA expression that results from a diminished $mTEC^{high}$ cell pool?*

2. *Does the lack of appropriate thymic negative selection during aGVHD cause an escape of autoreactive T cells to the periphery?*

Specific aim 2: To test whether preventing thymic donor T-cell infiltration via the $S1PR_1$ agonist KRP203 protects the TEC compartment and allows for normal thymic development and negative selection in experimental aGVHD.

Sphingosine-1-phosphate receptor 1 ($S1PR_1$) is critically involved in T-cell traffic and $S1PR_1$ agonism is known to prevent migration to GVHD target tissues.[181] Therefore, I intended to ask the following questions:

3. *Does KRP203 affect posttransplantation T-cell regeneration?*

4. *Does interference in the $S1PR_1$ pathway via KRP203 prevent alloreactive donor T-cell migration into the host thymus during aGVHD?*

5. *Does KRP203 able to prevent thymic epithelial cell injury and hence allows for normal thymic negative selection and avoid the escape of autoreactive T cells during aGVHD?*

6. *Is KRP203 able to preserve GVT activity?*

4. Materials & Methods

4.1 Mice

Female C57BL/6 (B6; H-2^b), C57BL/6.CD45.1 (B6Ly5.1; H-2^b), Balb/c (H-2^d), CBy.PL (B6)-Thy1^a/ScrJ (Balb/c-Thy1.1;H-2^d), B6.Cg-Tg(TcrαTcrβ)425Cbn/J (OT-II;H-2^b) and C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/WehiJ (RIP-mOVA;H-2^b), C57BL/6RAG$^{-/-}$ were bred in house but originally purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 x DBA/2 F_1 (BDF_1; H-$2^{b/d}$) were purchased from Janvier (Le Genest Saint Isle, France). 129/Sv (H-2^b) mice were purchased from Charles River (Lyon, France). All mice were kept in SPF conditions and in accordance with institutional regulations.

4.2 Allo-HSCT and GVHD induction

4.2.1 Myeloablative MHC mismatched allo-HSCT without aGVHD

To evaluate thymus-dependent T-cell reconstitution post allo-HSCT without GVHD, B6 Thy1.2 mice received total body irradiation (TBI = 1000cGy, in 2 series separated by 3 hours) and were reconstituted intravenously (i.v.) with 7x10^6 T-cell depleted bone marrow (TCDBM) from Balb/cThy1.1 mice. Mice were sacrificed at 1.5, 3 and 6 weeks post HSCT and thymus, spleen and LN were isolated, counted and analyzed by flow cytometry. To study thymic import and intrathymic T-cell development post allo-HSCT, B6 deficient for the recombinating activating gene (RAG$^{-/-}$) recipients were lethally irradiated (1000cGy) and reconstituted with 7x10^6 TCDBM from B6CD45.1^+ donors. Mice were analyzed 3 weeks post HSCT.

4.2.2 Unconditioned haplo-ID MHC mismatched allo-HSCT model with aGVHD

To investigate the direct effect of donor mature T-cell mediated alloresponses on thymic function in aGVHD 35x10^6 splenic T cells from C57BL/6 (H-2^b) mice were i.v. injected into unirradiated F_1 generation (BDF1 mice, H-2^{bd}) (unconditioned haplo-ID allo-HSCT model; *B6→BDF1* = H-2^b →H-2^{bd}; *b→bd*). These mice develop symptoms of aGVHD such as thymic injury, splenomegaly, skin inflammation and diarrhea within 2 weeks post-transplantation.[130,199,200]

4.2.3 Myeloablative MHC mismatched allo-HSCT model with aGVHD

To study aGVHD in a fully myeloablative MHC)-mismatched model, $7x10^6$ TCDBM from Balb/cThy1.2$^+$ (H-2^d) mice were co-transferred with $2x10^6$ purified splenic CD3$^+$ T cells (TCDBM + T cells) from Balb/cThy1.1$^+$ (H-2^d) donors into lethally (1000cGy) irradiated B6 (H-2^b) recipients (Balb/c→B6 = H-2^d→H-2^b; *d*→*b*). TCDBM alone served as a control. Mice were sacrificed at 2 and 4 weeks post-HSCT and spleen, LN and thymus were analyzed.

4.2.4 Myeloablative minor-HA mismatched allo-HSCT model with aGVHD

To study aGVHD in a miHA-mismatched model, $7x10^6$ TCDBM ± $4x10^6$ purified CD3$^+$ T cells from 129Sv/S1 (H-2^b) donors were transferred into lethally (1000cGy) irradiated C57BL/6CD45.1$^+$ (H-2^b) recipients. Here, the symptoms of aGVHD manifest within 4 weeks post-HSCT. Mice were sacrificed 4 weeks post-HSCT and spleen, LN and thymus were analyzed.

4.2.5 OTII→RIP-mOVA; Transgenic myeloablative MHC mismatched allo-HSCT model with aGVHD

To study *de novo* production of autoreactive T cells in mice with aGVHD, a transgenic mouse model was used: B6 RIP-mOVA mice (H-2^b) that express membrane-bound ovalbumin (mOVA; residues$_{139\text{-}385}$) as a surrogate self-antigen under control of the rat insulin promoter (RIP) in the pancreas but also in the thymus medulla were lethally irradiated and received TCDBM ± T cells from Balb/c (H-2^d) donors. After 4 weeks, mice that received T cells develop aGVHD including thymic damage. To study negative thymic selection, the *d*→RIP-mOVA^b recipients were re-irradiated 4 weeks after the 1st allo-HSCT and infused with syngeneic TCDBM from OTII mice mixed with B6 wild-type TCDBM in a 1:3 ratio. Transgenic OT II mice were bred with Rag2-deficient OT-II mice, generating transgenic Vα2Vβ5 TCR specific for OVA$_{323\text{-}339}$, with B6.SJL-Ptprc^{a}Pep3^b/BoyJ (B6.CD45.1;H-2^b) on a CD45.1$^+$ congenic background originally at the Benaroya Research Institute (Seattle, WA).[201] HSC-derived OTII$^+$ (Vα2$^+$Vβ5$^+$ T cells) will undergo normal thymic development and will be negatively deleted after OVA recognition.[202,203] Mice that developed aGVHD will have an impaired negative selection due to mTEC damage and thus are not able to delete OT-II$^+$ cells. The consequence is the release of *de novo* generated OVA-specific T cells into the periphery. Emergence and function of OVA-specific CD4$^+$ T cells (CD4$^+$CD45.1$^+$Vα2$^+$Vβ5$^+$) was tested after the 2nd syngeneic HSCT.

4.2.6 Myeloablative MHC mismatched allo-HSCT model with cGVHD

To study the development of cGVHD through thymic dysfunction, $0.1x10^6$ of $CD8^+$ T cells from C57BL/6 (H-2^b) donors were transferred into lethally (800cGy) irradiated Balb/c (H-2^d) recipients. In this model, auto-Ab are produced in skin and salivary glands within 60 days post-HSCT.[137]

4.3 KRP203 administration

4.3.1 Dose-response

Naïve WT C57Bl/6 mice were injected i.p. with 0.3mg/kg, 1mg/kg or 3mg/kg KRP203 every second day for 7 days to test dose/response relationships. KRP203 was initially dissolved in 0.5% methyl cellulose (MC) for oral gavage. As a control, MC alone was given orally. For i.p. injections, KRP203 was dissolved in PBS. Safety, tolerability, pharmacokinetics and efficacy of KRP203 in patients undergoing stem cell transplant for hematological malignancies will be evaluated in a parallel clinical study that had been initiated prior to initiation of our preclinical research (ClinicalTrials.gov Identifier: #NCT01830010). This interventional study is sponsored by Novartis Pharmaceuticals

4.3.2 Therapeutic administration

For therapeutic application, mice received 3mg/kg KRP203 i.p. every 2nd day starting 1 week after HSCT until the end of experiment.

4.3.3 Prophylactic administration

For prophylactic administration, mice were injected i.p. with 3mg/kg of KRP203, every 2nd day continuously from day-1 of transplantation until the end of experiment.

4.3.4 Withdrawal

For withdrawal experiments, mice received KRP203 at a dose of 3mg/kg from day -1 of transplantation every 2nd day for 7 or 14 consecutive days. Analysis of mice was either at day 14 or 21 respectively.

4.4 *In vivo* tumor injection

To induce aGVHD in mice carrying the A20 tumor, $7x10^6$ TCDBM from B6CD45.2$^+$ (H-2^b) mice were co-transferred with $2x10^6$ purified splenic CD3$^+$ T cells (TCDBM + T cells) from B6CD45.1$^+$ (H-2^b) donors into lethally irradiated (800cGy) Balb/c recipients (H-2^d) recipients (B6→Balb/c = H-2^b→H-2^d; *b→d)*. $1x10^4$ A20 luciferase$^+$ cells (this cell line was a present from Gang Zhou from Johns Hopkins University) were injected into the inguinal lymph node as described[204] one day post allo-HSCT under anesthesia with isoflurane. Mice recovered from anesthesia within 2min after the injection.

4.5 *In vivo* cell proliferation

To determine proliferation in different DN stages, mice were pulsed with BrdU [1mg/mouse, dissolved in PBS, 200ml injection volume] i.p. 3 hours and 1 hour before sacrificing the mice. To determine long-term proliferation, BrdU was diluted to 0.8mg/ml in the drinking water and mice received the drinking water for 1 consecutive week. Mice were sacrificed, organs isolated and BrdU$^+$ cells were determined by flow cytometry according to the manufactory's protocol (BD Pharmingen #559619 BrdU Flow Kits) and as published in Krenger *et al*. [130]

4.6 Cell preparation

4.6.1 Cell counting

To determine absolute cell numbers, organs were harvested and cell suspensions made. From the cell suspension, 50ml were taken aside and 5ml were acquired with the flow cytometer BD Accuri^{TM} C6 (BD Bioscience). The absolute cell numbers were calculated by multiplying acquired cell numbers with the dilution factor.
To determine absolute cell numbers in the PB, counting beads were used according to the manufacturer's protocol (Invitrogen, AccuCheck Counting Beads, #PCB100, Invitrogen)

4.6.2 T-cell enrichment

For T-cell enrichment, whole splenocytes were isolated from donors. Erythrolysis was performed with ACK buffer (self-made) for 3min. The cells were magnetically enriched with an anti-CD3 antibody according to the manufacturer's protocol (Invitrogen, #11413D Dynabeads Untouched Mouse T cell Kit) and used for further injection.

4.6.3 T-cell depletion

To obtain BM cells, bones (tibia and femur) were crushed with a mortar. To deplete T cells the cell suspension was incubated with hybridoma supernatant containing monoclonal Abs against CD4 (RL172), CD8 (31M), pan-Thy (T24) and/or Thy1.2 (HO-12-4-9) for 15min on ice. The cells were washed and incubated with complement (Cedarlane, #CL3051, Low Tox-M Rabbit complement) for 45min in a water bath at 37°C. The cells were washed, counted and used for injection.

4.6.4 Thymic epithelial cell enrichment

For TEC enrichment, the thymus was isolated from the mouse and put in digestion buffer (Liberase (Roche) and DNAse I (Roche)) for 5min at 37°C. The cell suspension was then pipetted up and down about 10-20 times. This step was repeated several times until all cells were in suspension. Cells were washed and stained with α-G8.8 biotin antibody for 20min on ice. Cells were washed and anti-biotin microbeads (Miltenyi, #130-090-485) were added for 15min in the fridge. The cells were washed again and positively selected with the AutoMACS Machine as already described[138].

4.7 Antibodies and flow cytometry analysis

Single cell suspension were prepared from different organs, cells were stained, acquired on Fortessa® (Becton Dickinson, Mountain View, CA) and analyzed with the FlowJo Software (Treestar). For surface staining, cell suspensions were stained for 30min on ice, for intracellular staining cells were stained according to the manufactory's script (Intracellular Fix/Perm Kit, eBioscience, # 88-8824-00). The murine monoclonal antibodies that were used are listed in the chapter 8: Reagents.

4.8 Immunohistochemistry

For the localization of $Aire^{+}$mTEC cells in the thymus, immunofluorescence microscopy was used. Therefore the organs were embedded in OCT and frozen with dry ice. The frozen organs were cut with a cryostat (Leica, CM1950) and fixed in acetone for 5 minutes. Samples were then rehydrated in PBS for approximately 5 min. The slide was dabbed on a tissue to remove the fluid. The slide was then incubated in blocking solution (PBS, 0.1% TritonX, 1% BSA, 1% goat serum) for 30 min. The samples were then washed and stained with the first antibody against cytokeratin

14 (CK14; rabbit, biotinylated) that is expressed by the medulla, for 60min at RT in blocking solution. In case of a directly labeled Ab (Aire) this step was skipped or blocking solution was added for 60min. After washing with PBS the slide was stained with the second Ab (goat anti-rabbit A488) or a directly labeled Ab for another 60min. After washing with PBS the slides were mounted with Hydromount and analyzed by fluorescent microscopy (Olympus BX61 Diana) or confocal microscopy.

4.9 Bioluminescence imaging

To detect bioluminescence of mice receiving luciferase$^+$ tumor cells, mice were injected i.p. with 150mg/kg Luciferin (Promega, #1605) 3 minutes before anesthesia and another 2min under anesthesia, before imaging. Imaging was performed with the NightOwl LB 983 in vivo Imaging System (Berthold Technologies). Peak signal intensity and tumor area were analyzed with the in vivo imaging software IndiGO (Berthold Technologies).

4.10 Statistical Analysis

All values are depicted as mean ± SD. Statistical analysis comparing two groups was performed using Mann-Whitney U test. Three groups or more were analyzed using Kruskal-Wallis one-way ANOVA and a Dunn's multiple comparison with the GraphPad Prism (GraphPad Software, La Jolla California USA). Pooled experiments were analyzed with two-way ANOVA and Fishers' LSD uncorrection. This uncorretion test is commonly used to exclude inter-experimental variations when pooled data were used. For P values > 0.05 = ns (not significant), $P \leq 0.05$ = *, $P \leq 0.01$ = **, $P \leq 0.001$ = ***.

5. Results

5.1 Impaired thymic expression of tissue-restricted antigens licenses the *de novo* generation of autoreactive CD4$^+$ T cells in acute GVHD

Our laboratory had previously generated data in experimental allo-HSCT systems which demonstrated that the thymus is an important target of donor T-cell alloreactivity during aGVHD.[129] This TRT weakens in consequence the platform for central tolerance induction as individual TRA are purged from the total repertoire due to a decline in Aire$^+$mTEC^{high}.[134] These data had provided a putative mechanism for how autoimmunity may develop in the context of antecedent alloimmunity. However, a formal proof of this contention was still lacking at the time of initiation of the present PhD thesis. Therefore, my first experiments - as part of a collaborative effort with the first author of this ongoing project - were aimed at demonstrating whether a direct link existed between a functional compromise of mTEC and the peripheral emergence of autoreactive T cells during aGVHD. Transgenic mice that express membrane-bound ovalbumin (mOVA) under the rat insulin promoter (RIP) were deemed suitable to address putative mechanistic links between altered thymic TRA expression and the thymic production of TRA-specific T cells in aGVHD. In these RIP-mOVA mice, OVA is expressed in the pancreas but also in the thymus specifically in mTEC as a membrane bound surface protein. mOVA thus mimics an unique surrogate self-antigen that can be subject to self-tolerance induction. Conversely, transgenic OT-II mice that are deficient for the recombinant activating gene (RAG) mice carry a TCR (Vα2Vβ5 chain) that is specific for the $OVA_{323-339}$ peptide. Therefore, to test whether loss of mOVA expression affected central deletion of OT-II OVA-specific T cells during aGVHD, we transplanted lethally irradiated RIP-mOVA mice (H-2^b) with TCDBM ± T cells from Balb/c (H-2^d) donors (Figure 8). After 4 weeks, mice that had received T cells developed aGVHD and thymic damage. After a secondary irradiation, TCDBM from OT-II mice were transplanted. In this system, HSC-derived Vα2$^+$Vβ5$^+$ OT-II T cells were expected to undergo normal thymic development and to be negatively selected after high-affinity OVA recognition in the thymus. In contrast, mice that developed aGVHD were expected to own an impaired negative selection due to mTEC damage and TRA (mOVA) loss. In consequence, OT-II T cells were not expected to be deleted to the same extent anymore and hence should be released into the periphery.

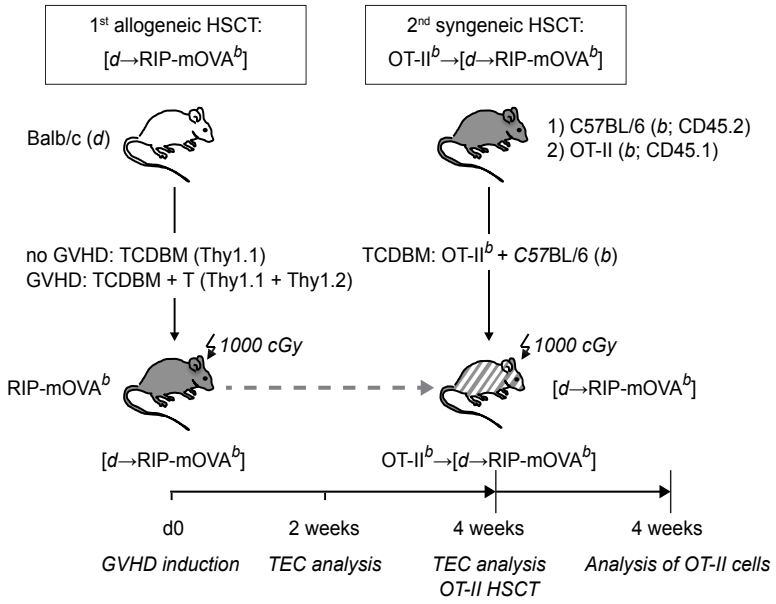


Figure 8: ***Mouse model to study thymic de novo generation of autoreactive T cells during aGVHD.*** *To induce acute GVHD (aGVHD), age-matched lethally irradiated RIP-mOVA recipients (H-2^b) received co-injection of TCDBM from Balb/cThy1.1$^+$ mice (H-2^d) + splenic T cells from Balb/c Thy1.2$^+$ (H-2^d) donors. Injection of TCDBM alone served as a control. 4 weeks after the 1st allo-HSCT, RIP-mOVA recipients got lethally re-irradiated and transplanted with TCDBM from OT-II CD45.1$^+$ and C57BL/6 CD45.2$^+$ donors. Another 4 weeks later, mice got sacrificed and thymus and secondary lymphoid organs were analyzed. Thymus-dependent autoreactive T cells were detected by the congenic marker CD45.1$^+$ together with the expression of the TCR chains Vα2$^+$Vβ5$^+$ that were expressed by the OTII$^+$ T-cell clones.*

All experiments were performed in 2014-2015. The data were published (Dertschnig et al., Blood 2015,125:2720, see Annex) and are hence only summarized here:

Our data consistently demonstrated a reduction in the expression of both Aire mRNA and protein and also mOVA RNA expression as a consequence of aGVHD-mediated TEC injury. Since Aire regulates OVA expression[205] and since the Aire$^+$mTEC^{high} subset is reduced in numbers during aGVHD,[134] our data argued that loss of Aire$^+$mTEC^{high} was responsible for the deficiency in thymic OVA during aGVHD.

We postulated that aGVHD interfered with negative selection of the OVA TCR because (a) Aire$^{-/-}$ RIP-mOVA mice cannot efficiently delete OT-II T-cells[205] and (b) total thymic mOVA expression levels correlate with deletion efficacy of OVA-reactive TCR.[104,203,205-207]

Thymic OT-II CD4$^+$ T-cell development was monitored by assessment of CD45.1$^+$ cells (Figure 8). An adequate ratio[206] between CD45.1$^+$ immature CD4$^+$CD8$^+$ (DP) and mature CD4$^+$CD8$^-$ thymocytes (CD4SP) indicated regular deletion of OVA-specific TCR in OT-II^b→[*d*→RIP-mOVA] mice without disease, as expected (see publication). Much lower DP/CD4SP ratios were observed in transgenic recipients with aGVHD (low thymic mOVA) however, indicating

inefficient deletion of OT-II cells. DP/CD4SP ratios were in the majority of these mice not distinguishable from ratios in OT-II^{b}→[*d*→C57BL/6] non-deleting controls (no thymic mOVA). Deficient elimination of OT-II cells in transgenic mice with aGVHD was substantiated by two- to three-fold higher frequencies of CD45.1^{+}CD4SP among total thymic CD4SP cells when compared to mice without aGVHD. Thus, an aGVHD-mediated loss of OVA expression in mTEC^{high} resulted in an unopposed escape of "forbidden" OVA-specific Vα2^{+}Vβ5^{+}CD4^{+} T-cell clones within the host thymus.[202] Importantly, higher numbers of OT-II cells were also present in LN and spleens of transgenic mice with aGVHD when compared to transgenic mice without disease. Since mature OT-II T cells were not passively transferred from donor grafts, formation of the peripheral OT-II pool was thymus-dependent.

Moreover, in transgenic recipients with aGVHD, the fraction of C57BL/6 (CD45.1^{-}) donor bone marrow-derived Foxp3^{+} T$_{regs}$ among total splenic CD4^{+} cells were reduced in frequency from a normal average of 10% to an average <1% (see Appendix, Manuscript Figure 2B). Among CD45.1^{-}Foxp3^{+} cells, some were FR4^{high}CD73^{high}, documenting their anergic phenotype. In contrast, emerging OT-II (CD45.1^{+}) cells were exclusively Foxp3^{-} conventional T cells whose FR4^{-}CD73^{-} phenotype suggested that they were non-anergic. Indeed, CD45.1^{+}CD4^{+} (OT-II) cells but not CD45.1^{-}CD4^{+} (non-OT-II) cells isolated from aGVHD mice vigorously responded to OVA peptide in culture (see Appendix, Manuscript Figure 2C and 2D).

Taken together, these results provided direct evidence in transgenic mice using OVA as model TRA that intrathymic *de novo* production of TRA-specific CD4^{+} T cells during aGVHD is triggered by impaired ectopic TRA expression. These OVA-reactive T cells are exported into a periphery that is characterized by T$_{reg}$ deficiency. Hence, the functional compromise of the mTEC compartment may provide a pathogenic link between alloimmunity and the development of autoimmunity.

Our results published in Blood led me to propose that interference with alloreactive donor T-cell trafficking to the host thymus might prevent thymic damage. Protection of the mTEC compartment, including TRA-expressing Aire^{+}mTEC^{high} could in turn prevent breakdown of thymic central tolerance induction. A successful intervention could hence avert the export of autoreactive T cells to the periphery and therefore block the pathogenic mechanism responsible for the transition of aGVHD to the chronic form of disease. Since S1PR$_1$ is known to be critically involved in lymphocyte trafficking, I hypothesized that the specific S1PR$_1$ agonist KRP203 could achieve this goal. Using experimental allo-HSCT models, I tested this hypothesis in my subsequent work.

5.2 The influence of KRP203 on thymus-dependent T-cell regeneration in the absence of aGVHD

5.2.1 Continuous KRP203 administration reduces T-cell numbers circulating in the blood of untransplanted mice

The administration of KRP203 may offer an effective novel modality to prevent human GVHD by inhibiting alloreactive T-cell trafficking from SLO into peripheral tissue.[181] Gaps in knowledge remain, however, with regard to KRP203-mediated $S1PR_1$ agonism on posttransplantation intrathymic T-cell development and thymic export of new naïve T cells. Since $S1PR_1$ activation is required for the export of new naïve T cells from the thymus,[163,164] any intervention intended for GVHD prophylaxis may also alter the magnitude of posttransplantation thymic T-cell production and therefore impair T-cell regeneration. I addressed this importation issue in my first experiments using naïve mice and non-GVHD transplantation models, which are uniquely suited for independent manipulations of different experimental variables and to assess the efficacy and pharmacodynamics of KRP203.

Novartis Inc. has previously generated preclinical data to describe the pharmacodynamics and toxicology of KRP203,[197] which were not required to be repeated in the present thesis. KRP203 has been shown to effectively redistribute lymphocytes to SLO and thus prevents effector cell migration to the target organs in various animal models of autoimmune disease.[196] Nevertheless, to confirm the efficacy of the drug in the specific murine models studied during the present PhD project, I chose to initiate experiments using naïve mice. To establish a dose-response relationship, I firstly injected wild-type C57BL/6 mice i.p. with 0.3mg/kg, 1mg/kg or 3mg/kg KRP203 dissolved in 0.5% methyl cellulose (MC) every second day based on published protocols[208,195,188]. As a control, 0.5% MC alone was injected. On day 7 after continuous KRP203 administration, mice that received 3mg/kg had the lowest lymphocyte counts in the PB (25±15% reduction), in comparison to the group that received 1mg/kg (15±7%) or 0.3mg/kg (11±3%) (Figure 9A). Since the $S1PR_1$ is strongly expressed on T cells, I tested whether the reduction was true for both $CD4^+$ and $CD8^+$ T cells after KRP203 administration for 2 weeks. Indeed, I observed a decrease for $CD4^+$ (from $3.5\pm1.2x10^4$ to $1.5\pm1.2x10^3$) and $CD8^+$ T cells (from $2.9\pm1.5x10^4$ to $8.7\pm6.1x10^2$) among whole peripheral blood mononuclear cells (PBMC) (Figure 9B). To determine the pharmacokinetics of KRP203, PBMC from B6 mice were measured during a 14-day treatment period. During that time, mice received 3mg/kg KRP203 i.p. every 2nd day for 11 days. KRP203 application was then withdrawn (WD) for another 4 days (Figure 9C). Lymphocyte counts were significantly reduced in the PB between day 1 and 2 after the first injection (from $\leq1x10^4$ to $\leq1x10^2$ lymphocytes/ml) and remained so for 11 days until the drug

was withdrawn. Since the ultimate goal was to apply KRP203 in an allo-HSCT setting, this was an important observation and strongly advocated the use of a prophylactic KRP203 regimen (i.e. start one day before transplantation (day-1)) in order to reach an optimal efficacy in lymphocyte migration reduction. Importantly, two days after WD, lymphocyte counts returned to numbers present in normal control B6 mice ($\leq 1x10^4$ lymphocytes/ml) (Figure 9D). These results indicated that KRP203 administration was best applied continuously (every second day). Based on these data, I decided to use a standard KRP203 administration schedule for future experiments that consisted of i.p. injections of the highest dose tested (3 mg/kg) continuously (meaning every 2nd day) from day-1 until the end of a given experiment.

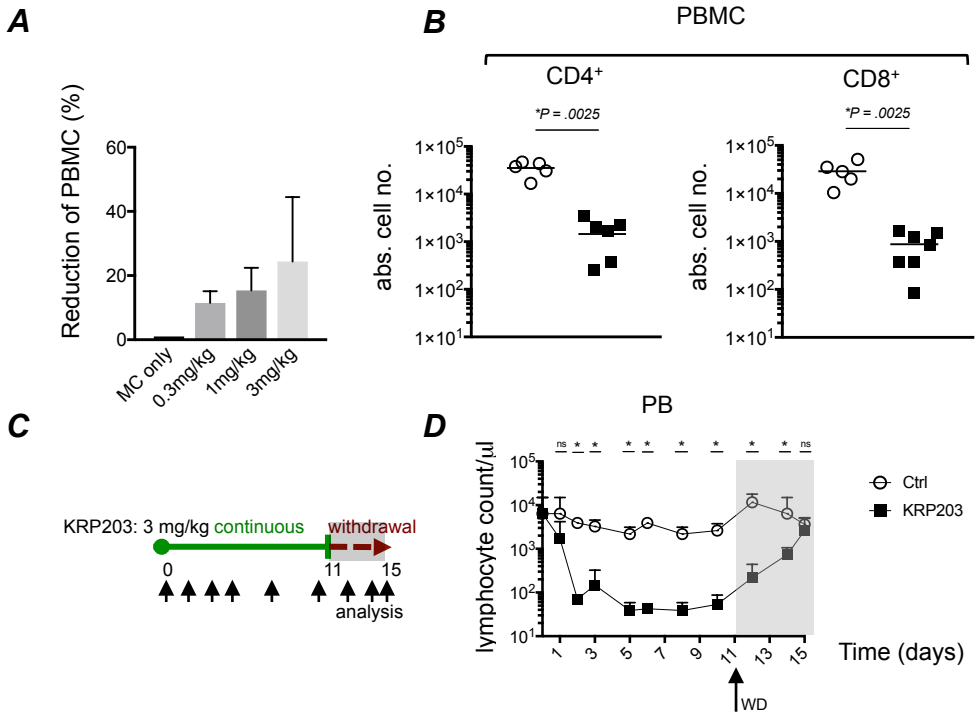


Figure 9: ***Continuous KRP203 administration reduces T-cell numbers in the peripheral blood (PB) of naïve mice in a dose-dependent manner.*** *Reduction (in %) of peripheral blood mononuclear cells (PBMC) is shown as a function of the applied KRP203 dose after 7 days of continuous (every 2nd day) i.p. injection of 0.3mg/kg, 1mg/kg or 3mg/kg KRP203. Control mice received methyl cellulose (MC) alone (A). Absolute cell numbers of CD4+ and CD8+ T cells among whole PBMC are shown at 2 weeks after continuous KRP203 application (3mg/kg, i.p., every 2nd day) (■). Untreated control mice received MC only (O) (B). Experimental scheme of KRP203 administration (C). Lymphocyte counts from peripheral blood (PB) were measured over 15 days during continuous KRP203 injection and after withdrawal (WD) at day 11 (D). This figure represents pooled data from independent experiments with ≤3 mice per group. *p< 0.05, Mann-Whitney U-test.*

5.2.2 KRP203 increases frequencies of CD4SP and CD8SP T cells in the thymus of untransplanted mice

The ultimate goal of my experiments was to establish whether KRP203 - via interference with alloreactive donor T-cell migration to the thymus - would limit aGVHD-mediated thymic injury and hence improve thymus-dependent T-cell regeneration following allo-HSCT. The effects of $S1PR_1$ agonism on posttransplantation intrathymic T-cell development and thymic export of new naïve T cells have not been investigated to date. Since $S1PR_1$ activation is required for both the import of progenitors to and export of new T cells from the thymus in normal mice,[163] any intervention intended for aGVHD prophylaxis via KRP203 may, however, also unintentionally lower the magnitude of thymic T-cell production and hence impair posttransplantion T-cell regeneration. To approach this possible problem, my first experiments were aimed at establishing the effects of KRP203 on intrathymic T-cell development in normal untransplanted mice. To this end, I injected KRP203 (3mg/kg) i.p. every 2nd day in naïve mice. After 1 week, mice were sacrificed and the thymic T-cell compartment was analyzed by flow cytometry. The CD4,CD8 double negative (DN) compartment was not affected by KRP203 with regard to absolute cell numbers (Figure 10A, upper row) and frequencies (Figure 10B, upper row). In contrast, the CD4,CD8 double positive (DP) compartment showed a significant decrease in its frequency (mean from 82.7±3.6% to 70.6±5.8%, ****P=0.0001*) when mice received KRP203 for 1 consecutive week (Figure 10B, upper row). The decrease of the DP cell compartment was associated with a concomitant increase of CD4SP cells with regard to absolute cell numbers ($4.8\pm1.6x10^6$ to $1.4x10^7\pm3.4x10^6$ ****P= <0.0001*) and frequencies (from 8.8±2.7% to 18.1±4.6%, ****P=0.0001*). Similarly CD8SP cell numbers increased from $2.1x10^6\pm8.1x10^5$ to $3.9\pm1.3x10^6$, ****P=0.0057* and frequencies were also higher from 3.9±1.7% to 7.2±1.5%, ****P=0.0008* upon KRP203 administration (Figure 10A, B, respectively, lower panel).

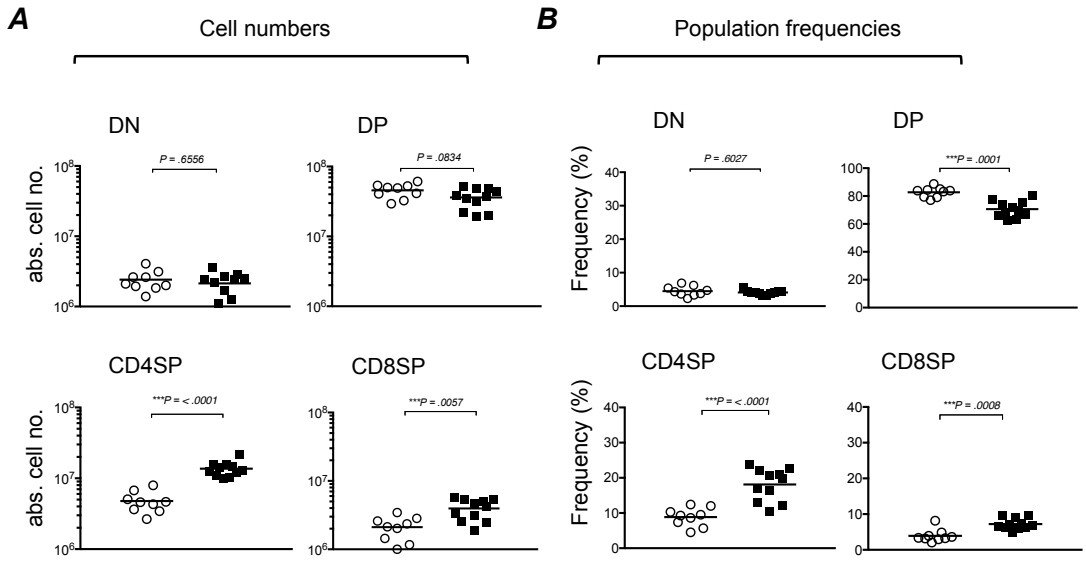


Figure 10: ***KRP203 increases cell numbers and frequencies of CD4SP and CD8SP T cells in the thymus of naïve mice****. Naïve female B6 mice received KRP203 (3mg/kg, i.p., every 2nd) day for 2 consecutive weeks. Absolute cell numbers (A) and frequencies (B) of CD4SP, CD8SP, DN and DP T cell subsets were determined in the thymus from naïve mice with KRP203 application (■) or without (O). These data represent combined data from 3 independent experiments with n=3 mice per group. Endothelial cells (CD45$^-$CD31$^+$) in the thymus were stained for P-Selectin expression 1 week with (■) and without (O) KRP203 injections and absolute cell numbers were determined from one representative experiment (C). *p< 0.05, Mann-Whitney U-test.*

This increase of SP cells suggested an accumulation of T cells upon KRP203 administration that might be due to a block of thymic export as it was previously reported for FTY720.[163] It was previously shown in mice that if the export of thymocytes is blocked, CD4SP and CD8SP T cells fill up the niches in the thymus over time that in turn leads via a feedback mechanism to a block in thymic import.([209 where S1P is suggested to act as a feedback signal.[210] Thus, diminished thymic import has no or reduced expression of P-Selectin (CD62P) on endothelial cells acting as a recruitment signal for thymic progenitors.[210] Thus, to answer the question if KRP203 might affect the export of thymocytes, I measured P-Selectin expression on CD31$^+$ endothelial cells in the thymus by flow cytometry and calculated absolute cell numbers. I did not observe a significant decrease in CD31$^+$P-Selectin$^+$ cells (Figure 11A) although there was a trend towards lower P-Selectin expression (albeit not statistically significant). Decreased P-Selectin expression indeed suggested the presence of full niches within the thymus. However, my results did not provide conclusive results if the accumulation is indeed due to a blocked thymic export. Therefore, I further tested if the accumulation was a consequence of increased proliferation. To address this question, I injected naïve B6 mice with KRP203 for 3 days and pulsed them with BrdU 3 hours and 1 hour before analysis. BrdU incorporates into the DNA and can be detected with α-BrdU Ab by flow cytometry in proliferating cells. I did not observe a significant

difference in proliferation. Nevertheless, a trend towards higher proliferation upon KRP203 administration in all T-cell subsets was observed (CD4SP, CD8SP, DP and DN) (Figure 11B from left to right respectively). These data led to the tentative conclusion that both – enhanced proliferation and blocked export – may have been causes for the accumulation of SP cells. This contention, however, needs to be examined in more detail in studies not performed in this thesis.

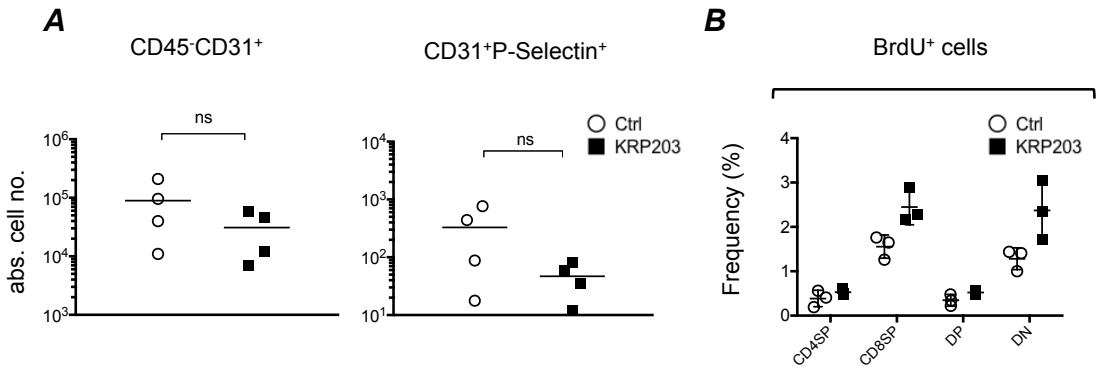


Figure 11: ***Accumulation of CD4, CD8 single positive cells might be due to full niches and increased proliferation****. Naïve female B6 mice received KRP203 (3mg/kg, i.p., every 2nd) day for 7 days. Thymic endothelial cells (CD45- CD31+) were stained for P-Selectin expression 1 week with (■) and without (O) KRP203 injections and absolute cell numbers were determined (A). These data represent 1 experiment with n=4 mice per group. *p< 0.05, Mann-Whitney U-test. Naïve female B6 mice received KRP203 (3mg/kg, i.p., every 2nd day) for 3 days. Mice were pulsed with BrdU i.p. 3 hours and 1 hour before analysis. Frequencies of BrdU+ subsets were determined from naïve mice with KRP203 application (■) or without (O) (B). These data represent 1 experiment with n=3 mice per group.*

Taken together, these results raised the possibility whether KRP203 inadvertently influenced thymus-dependent T-cell production post-HSCT in a negative way. To address this problem, I next tested whether and how posttransplantation thymus-dependent T-cell regeneration was indeed affected in transplanted mice without aGVHD.

5.2.3 Thymic T-cell maturation is normal in allo-HSCT recipients under the KRP203 umbrella

I tested whether $S1PR_1$ agonism via KRP203 had an inhibitory effect on the export of newly generated naïve T cells from the thymus to the periphery in mice receiving allo-HSCT but in the absence of thymic injury induced by alloreactive donor T cells. To this end $7x10^6$ TCDBM from Balb/c donors were transplanted into lethally irradiated MHC-mismatched B6 recipients (*Balb/c→B6* = H-2^d→H2^b; *d→b*). KRP203 was administered prophylactically (day-1) and continuously until the end of the experiment (1.5, 3 and 6 weeks). Mice were sacrificed 1.5, 3 and 6 weeks after transplantation and the thymus was analyzed. Donor BM-derived T cells could be

detected with the congenic marker Thy1.1 (CD90.1) that was exclusively expressed by donor cells (Figure 12A). Different thymic subsets including CD4SP, CD8SP, DP and DN were determined by flow cytometry and absolute cell numbers were counted. The data demonstrated that all T-cell subsets were comparable to the untreated control mice with regard to absolute cell numbers at all time points analyzed (Figure 12B).

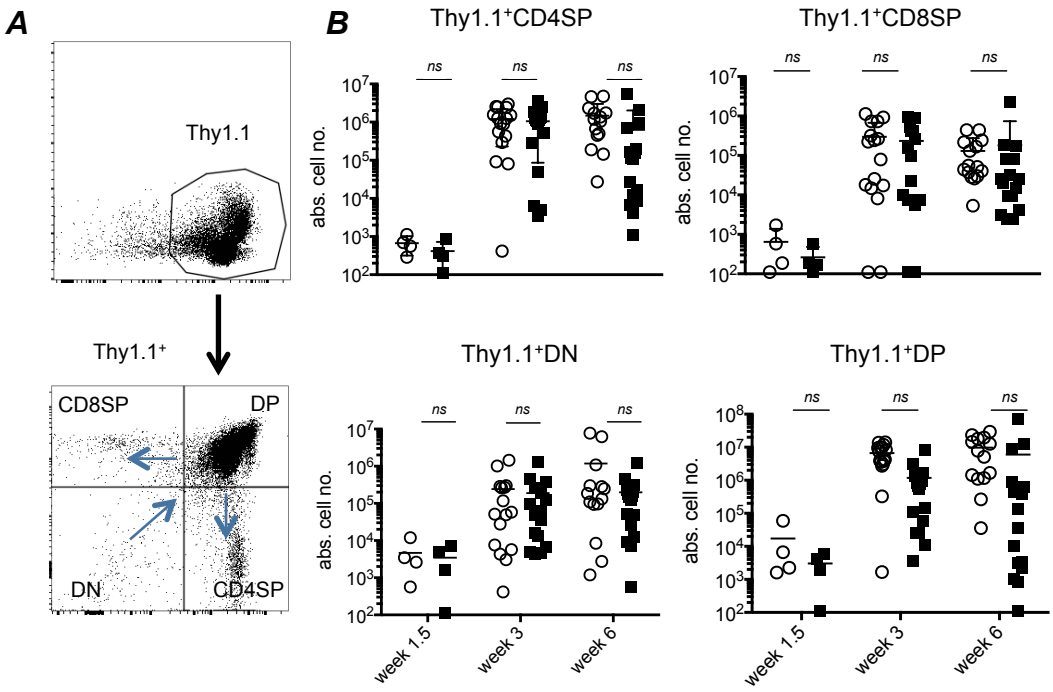


Figure 12: ***Thymic T-cell maturation is normal in allo-HSCT recipients in the presence of KRP203.*** *Lethally irradiated B6 recipients received TCDBM without (O) and with (■) KRP203 administration (3mg/kg, i.p., ever 2nd day) day-1 before allo-HSCT until 1.5, 3 and 6 weeks post-HSCT from a fully MHC-mismatched (H-2^d→H-2^b) Balb/c donor. Reconstituted donor BM-derived T cells were identified by the expression of the congenic marker Thy1.1 (A). The absolute cell numbers of the thymus were determined at 1.5, 3 and 6 weesk post allo-HSCT (B). The graphs represent data from 4 independent experiments with n=4 mice per group. *p< 0.05, Mann-Whitney U test.*

To evaluate the effect on early thymocyte development, I determined absolute cell numbers of DN stages 1-4 (DN1-DN4; CD44$^+$CD25$^-$, CD44$^+$CD25$^+$, CD44$^-$CD25$^+$, CD44$^-$CD25$^-$ respectively). DN1, DN2 and DN4 were not affected by KRP203 as cell numbers were comparable at 3 weeks post allo-HSCT in both cohorts. However, the DN3 and an intermediate DN3-4 stage showed a significant decrease of absolute cell numbers when mice received KRP203 (Figure 13A). This observation raised the question whether KRP203 affected proliferation of the thymocytes within these particular stages. To address this question, I applied BrdU in the drinking water for 1 consecutive week. The frequencies of BrdU$^+$ cells within the different DN subsets did not show any significant differences in mice that received KRP203 and those that did

not (Figure 13B). Next, I aimed to quantify thymic import in response to KRP203. To evaluate the import of ETP, I took advantage of lethally irradiated recombinant activating gene deficient ($RAG^{-/-}$) mice. Thymocytes of these mice are not able to develop further than the DN stage. Thus, I could specifically focus on the early thymic development in the absence of any influence or feedback mechanism of mature T cells. Therefore, I transplanted $7x10^6$ TCDBM from $B6CD45.1^+$ donors into lethally irradiated MHC-mismatched $B6RAG^{-/-}$ recipients. At 3 weeks post-HSCT I determined absolute cell numbers of ETP that have recently entered the thymus and which express the following markers[91]: $Lin^-CD44^+c\text{-}Kit^+Sca\text{-}1^+$ (Figure 13C). The Lin-cocktail contained CD4, CD8, CD11b, CD11c, CD19 and CD25. The data clearly demonstrated that there was no significant differences in ETP numbers and frequencies between mice with or without KRP203 (Figure 13C).

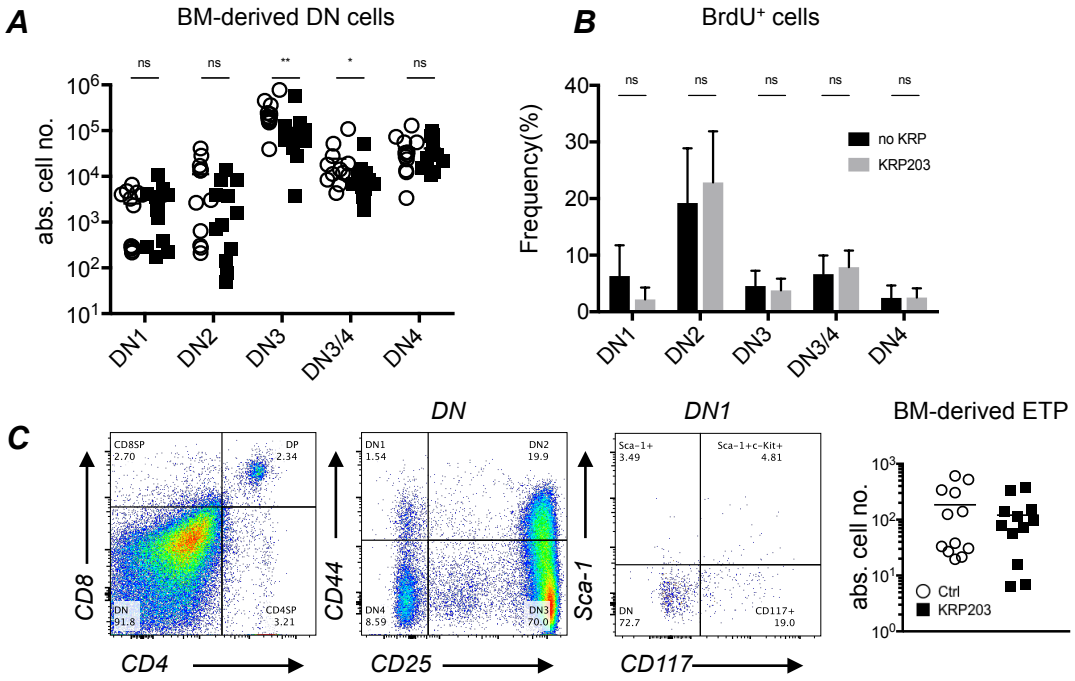


Figure 13: ***Intrathymic T-cell development is normal post allo-HSCT in the presence of KRP203.*** *Cells were harvested from the thymus of lethally irradiated B6 recipients that received TCDBM alone (○) or TCDBM + KRP203 (3mg/kg, i.p., every 2nd day) day-1 before allo-HSCT until the end of experiment (■) from MHC-mismatched Balb/c donors at 3 weeks post allo-HSCT. Absolute cell numbers were determined and analysis of ($CD4^-CD8^-$) DN stages 1-4 was performed by the surface expression of $CD44^+CD25^-$, $CD44^+CD25^+$, $CD44^-CD25^+$, $CD44^-CD25^-$ respectively (A). Proliferation was measured by BrdU administration in the drinking water for 1 week. $BrdU^+$ DN cells are illustrated as frequencies. (B). Early thymic progenitors (ETP) were identified by gating on Lin- (Lin: CD4,CD8,CD11b,CD11c,CD19,CD25) and $CD44^+c\text{-}Kit^+Sca\text{-}1^+$ cells. Absolute ETP numbers were determined in the thymus from lethally irradiated B6 recipients that were reconstituted by TCDBM from $RAG^{-/-}$ mice with (■) and without (○) KRP203 administration for 3 weeks (C).These data represent combined data from three independent experiments with n=4 mice per group. *p< 0.05, Mann-Whitney U test.*

In conclusion, I found that prophylactic administration of KRP203 followed by continuous administration did not impair the intrathymic T-cell maturation process after allo-HSCT in the absence of TRT.

5.2.4 Peripheral T-cell compartment is normal in allo-HSCT recipients under the KRP203 umbrella

As the thymocyte numbers did not show a significant difference upon KRP203 administration compared to control mice, I next investigated the peripheral T-cell compartment in LN and spleen in the same mouse model (*Balb/c→B6* = H-2^d→H2^b; *d→b*). KRP203 was administered prophylactically (day-1) and continuously until the end of the experiment. Mice were sacrificed 1.5, 3 and 6 weeks after transplantation and SLO were analyzed by flow cytometry and absolute cell numbers counted. I did not observe any significant differences in absolute cell numbers of BM-derived $CD4^+$ and $CD8^+$ T cells between mice that received KRP203 and control mice in the peripheral LN nor in the spleen (Figure 14A and B respectively). These results indicated that during KRP203 exposure there was no impairment of thymic export of BM-derived and *de novo* generated T cells into the periphery.

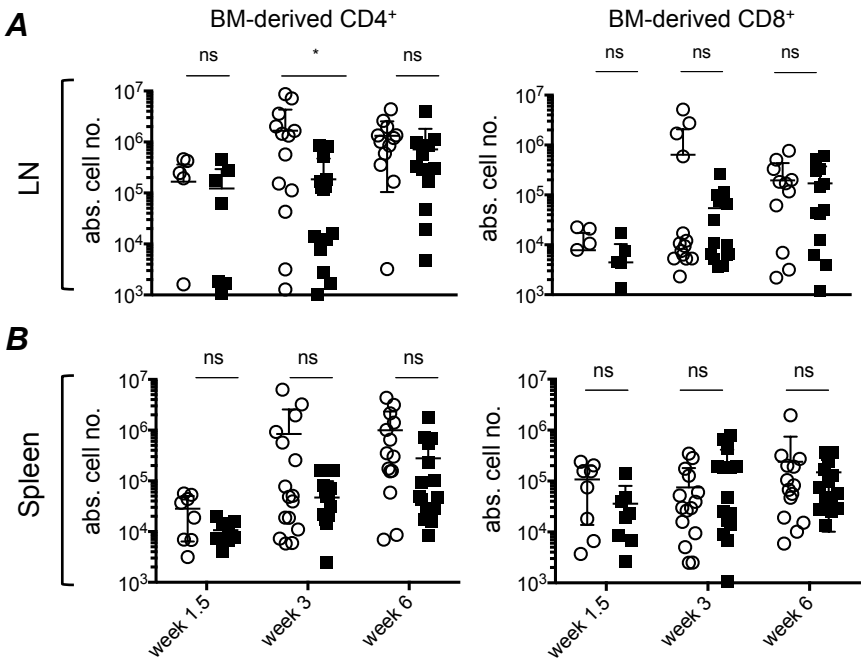


Figure 14: ***Peripheral T-cell compartment is normal post allo-HSCT in the presence of KRP203.*** *The peripheral lymphocyte compartment was analyzed of lethally irradiated B6 recipients that received TCDBM alone (○) or TCDBM + KRP203 (3mg/kg, i.p., every 2nd day) day-1 before allo-HSCT until the end of experiment (■) at 1.5, 3 and 6 weeks post allo-HSCT from MHC-mismatched Balb/c donors. Cells were harvested from peripheral lymph nodes (pLN) (A)*

*and spleen (B) and absolute cell numbers of CD4$^+$ and CD8$^+$ T cells were determined. The graphs represent data from 4 independent experiments with n=4 mice per group. *p< 0.05, Mann-Whitney U test.*

Taken together, my data gained in an allo-HSCT model did not confirm a previously published finding that thymic export is negatively affected by S1P modulation using FTY720 in untransplanted mice.[211] I therefore concluded that KRP203 did not have a detrimental effect on thymic export of newly developed T cells and in result allowed for the regeneration of a normal peripheral T-cell compartment with regard to its total cellularity following allo-HSCT.

5.3 The influence of KRP203 on intrathymic T-cell development in mice with aGVHD

5.3.1 Continuous prophylactic but not therapeutic KRP203 application reduces donor T-cell infiltration into the thymus during aGVHD

Having established that KRP203 did not negatively affect thymus-dependent T-cell regeneration in the absence of aGVHD, I then proceeded to test the effects of this drug in aGVHD where the use of $S1PR_1$ agonist KRP203 had been proposed to constitute a safe and effective novel modality for disease prevention.[197] Entry of pro-inflammatory donor T cells is a characteristic of aGVHD-induced thymic injury.[130] However, interference with alloreactive donor T-cell trafficking might prevent thymic damage. This hypothesis was tested in the following experiments. I used a well-studied unconditioned haplo-ID allo-HSCT model (*B6→BDF$_1$* = H-2^b → H-2^{bd}; *b→bd*).[130] I chose to use this particular model since it helps to decipher mechanisms that cannot be studied in more complex models that more closely reflect the clinical situation. Control mice received 35x10^6 splenocytes from a syngeneic donor *(bd→bd)* and did not develop aGVHD. Conversely, the transfer of 35x10^6 splenocytes from an allogeneic B6CD45.1$^+$ donor into BDF1 recipients *(b→bd)* induced aGVHD that was manifested in splenomegaly, weight loss and thymic loss of cellularity within 2 weeks (data not shown). The third group received allogeneic T cells together with continuous KRP203 administration *(b→bd + KRP203)*. Based on the fact that efficient lymphocyte reduction was reached 1-2 days post injection (Figure 9), I started KRP203 administration at day-1 before allo-HSCT and injected mice until the end of experiment. At 2 weeks after T-cell transfer, mice were sacrificed and thymic donor T-cell infiltration was analyzed by flow cytometry. To distinguish donor mature T cells from host T cells, I used the surface expression of CD45.1, a congenic marker that was exclusively expressed by donor mature T cells (Figure 15A, left panel). Among the host (Figure 15, middle panel) and donor compartments (Figure 15, right panel) I further gated on CD4$^+$ and CD8$^+$ T cells in order to calculate absolute cell numbers of the corresponding populations. Prophylactic KRP203

administration led to a significant reduction of absolute donor T-cell numbers infiltrating the recipient thymus at 2 weeks after T-cell transfer in comparison to mice with aGVHD but without KRP203 administration (from 1.5±1.2x10^5 to 2.2±1.8x10^6 respectively, ***P=0.0091*) (Figure 15B, upper graph). Alloreactive donor T cells attacked host thymocytes in the absence of KRP203, resulting in a decrease of absolute thymic cell numbers at 3 weeks after aGVHD induction. Thymic cellularity was preserved, however, when KRP203 was given (data not shown). In addition, donor T-cell numbers did not increase over time by continuous KRP203 administration (Figure 15B, lower panel). Moreover, the host CD4-, CD8-DP compartment was increased (60±5%) when KRP203 was given prophylactically in comparison to mice with aGVHD (48±20%) (Figure 15C). This reduction of DP in mice that received KRP203 in comparison to the control group (82±5%) only arose from the fact that CD4-, CD8SP cells increased in the presence of KRP203 administration which was compensated by a decreased frequency of DP. The decrease of alloreactive donor T-cell infiltration was mirrored in a fully myeloablated MHC-mismatched model, where I co-transferred 7x10^6 TCDBM + splenic T cells (TCDBM + T cells) from Balb/c (Thy1.2^+, Thy1.1^+ respectively) donors into B6 recipients in order to induce aGVHD, (*Balb/c→B6* = H-2^d→H-2^b; *d→b*). TCDBM alone served as a control. Thymus-infiltrating donor T-cell numbers were reduced by ≥1log (1.5±1.2x10^5 to 1.6±2.6x10^4, *P=0.0933*) when mice received prophylactic KRP203 (Figure 15D, left graph). The same was true for a minor-HA mismatched mouse model, where I co-transferred 7x10^6 TCDBM ± 4x10^6 splenic T cells from 129Sv/1 donors into B6Ly5.1 recipients. Absolute cell numbers of donor T cells were decreased from 3.4x10^5±9x10^4 to 1.1x10^5± 4.6x10^4 (Figure 15D, right graph).

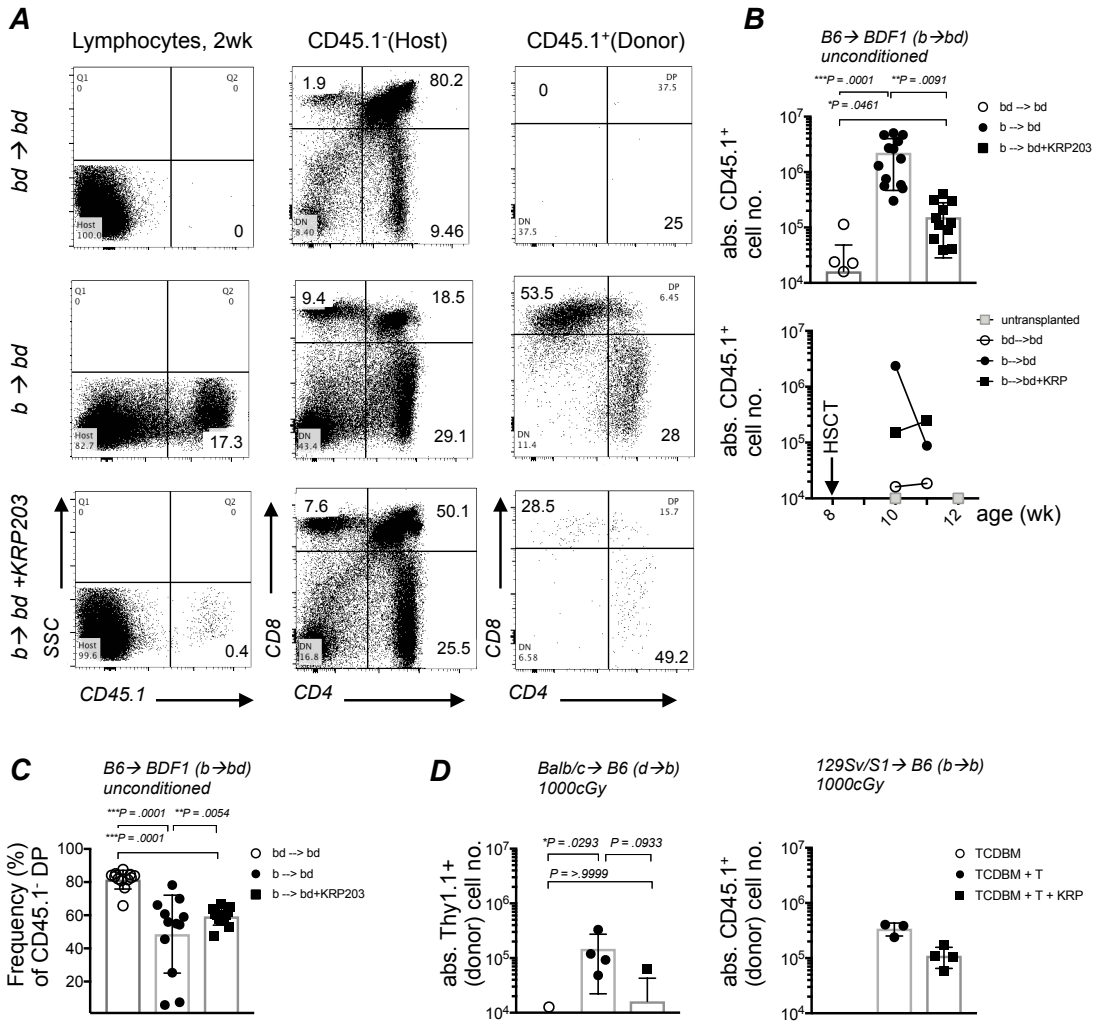


Figure 15: ***Thymic donor T-cell infiltration is reduced by KRP203 in the presence of acute GVHD.*** *Acute GVHD (aGVHD) (b→bd) was induced in 8-week old female BDF1 recipients by injection of splenic T cells from B6 donors. The gating strategy is displayed as FACS plots from one representative experiment. Donor T cells express the congenic marker CD45.1 (left column). The middle column shows the T-cell compartment of the host thymus (CD45.1$^-$) and the right column shows donor mature CD4 and CD8 T cells (A). Donor T-cell infiltration (CD45.1$^+$) was analyzed in mice without aGVHD (○), mice that developed aGVHD (●) and mice that received T cells and KRP203 (3mg/kg, i.p., every 2nd day from day-1 until the end of experiment) (■). Donor T-cell infiltration at 2 weeks is given as absolute cell numbers among total thymic cells from a haplo-ID unirradiated model (b→bd) that is depicted in the upper graph and absolute cell numbers of thymus-infiltrating donor T cells were determined over time at 2 and 3 weeks after T-cell transfer (b→bd) (B, lower graph). Host CD4, CD8 double positive (DP) compartment is shown in frequencies at 2 weeks post allo-HSCT (C). Donor T-cell infiltration (CD90.1$^+$) was analyzed in mice without aGVHD (○), mice that developed aGVHD (●) and mice that received T cells and KRP203 (■) (3mg/kg, i.p., every 2nd day from day-1 until the end of experiment) from a major-HC mismatched lethally irradiated model (d→b) which is shown in the left graph (D) and from a minor-HA mismatched mouse model (D,right graph) at 2 weeks post allo-HSCT. *p< 0.05, Kruskal-Wallis and Dunn's multiple comparison test.*

These data showed that prophylactic KRP203 administration could indeed diminish infiltration of pro-inflammatory donor T cells into the host thymus after allo-HSCT in mice with aGVHD.

5.3.2 Prophylactic but not therapeutic KRP203 application reduces thymic donor T-cell infiltration during aGVHD

To study not only the prophylactic but also the therapeutic effectiveness of KRP203 in mice that had already developed aGVHD, I used the same murine aGVHD, haplo-ID non-conditioned model as described before (*B6*→*BDF1* = H-2^{b} → H-2^{bd}; *b*→*bd*). Here, I included a fourth group: 1 week after T-cell transfer (day 7) mice received 3mg/kg KRP203 for another week. Totally 2 weeks after T-cell transfer, mice were sacrificed and PB and thymus were analyzed (Figure 16A). In the PB, absolute cell numbers of CD45.1^{+} donor mature T cells were counted. As expected, mice that developed aGVHD had high numbers of donor T cells present in the PB, whereas prophylactic KRP203 administration led to a reduction of CD4^{+} (from 7.2±7x10^{4} to 3.6±3x10^{3}) and CD8^{+} (from 1.9±3.4x10^{6} to 8.3x10^{3}±1.1x10^{4}) donor mature T cell counts in the PB. In contrast, the therapeutic administration did not result in reduction of T-cell numbers in the PB for CD4^{+} T cells (1.5±1.7x10^{4}) and CD8^{+} (7±7.4x10^{5}) (Figure 16B). The diminished T-cell migration upon prophylactic KRP203 administration could be confirmed by a reduction of ≥1log of CD45.1^{+} donor T cells in the thymus. In comparison, mice that received therapeutic KRP203 7 days after T-cell transfer even showed an increase of donor T cells present in the thymus (Figure 16C). These data indicated that therapeutic KRP203 administration resulted in an abundant number of alloreactive T cells in the thymus during aGVHD. These observations were highly relevant for potential use of KRP203 in the clinics as they helped to establish a suitable drug administration protocol.

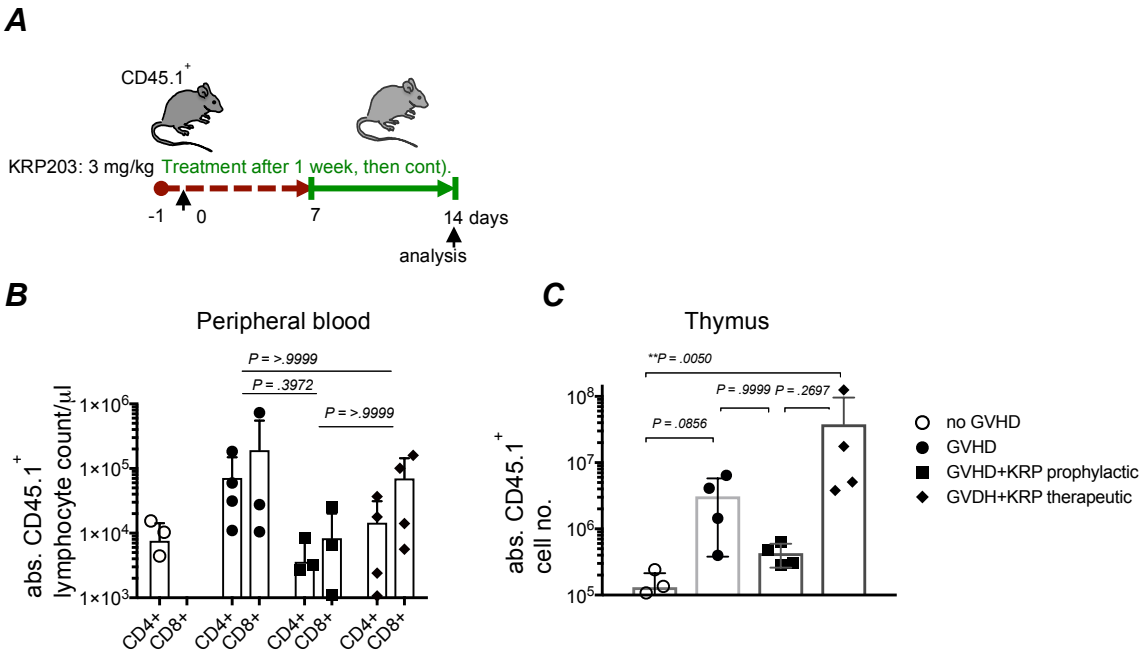


Figure 16: ***Prophylactic but not therapeutic KRP203 application reduces donor T-cell migration during aGVHD*** *Acute GVHD (aGVHD) (b→bd) was induced in 8-week old female BDF1 recipients by injection of splenic T cells from B6 donors. The approach for the KRP203 application is illustrated in (A): Mice without GVHD (O), with GVHD (●), with GVHD and prophylactic (start day-1 until the end of experiment) KRP203 administration (■) and therapeutic KRP203 application (start day 7 after T-cell transfer until the end of experiment) (◆). Both groups received the same dose and application form of KRP203 (3mg/kg i.p. every 2nd day) (A). Absolute donor T-cell numbers (CD45.1+) were determined 2 weeks after T-cell transfer in the PB (B) and in the thymus (C) by flow cytometry and cell counting of the appropriate population. *p< 0.05, Kruskal-Wallis and Dunn's multiple comparison test.*

5.3.3 The inhibitory effect of KRP203 on thymic donor T-cell infiltration is maintained for several weeks after drug withdrawal

An observation that is likely to be clinically relevant is the effect on donor T-cell migration after KRP203 withdrawal (WD) in the presence of aGVHD. Therefore, I used the same model as described above (*B6→BDF$_1$* = H-2^b → H-2^{bd}; *b→bd*). To investigate the effect after WD, mice received prophylactic KRP203 administration (day-1 until end of experiment), KRP203 for 2 weeks followed by WD for 1 week; and KRP203 for 1 week followed by WD for 2 weeks. As controls, mice without KRP203 or mice without aGVHD were analyzed. Mice were analyzed at 3 week after T-cell transfer (Figure 17A). Notably, one and 2 weeks after KRP203 WD, frequencies of donor T cells infiltrating the thymus remained low in comparison to mice that did develop aGVHD without KRP203 (1±0.5% to 17±1% respectively). The frequency of thymus-infiltrating donor T cells was comparable to frequencies in mice that continuously received KRP203 (1±0.5%) (Figure 17B). Importantly, lack of intrathymic inflammation allowed for normal T-cell development to occur in mice with KRP203, which was characterized by normal DP numbers

($1.6x10^7 \pm 1.1x10^7$) (Figure 17C). In parallel, the size of the $Aire^+mTEC^{high}$ compartment remained normal ($1.5 \pm 1.4x10^3$) (Figure 17D) which will be further discussed in chapter 5.4. In collaboration with Phil Smith (Novartis Inc.) I could confirm these data also in another mouse model where mice developed symptoms of cGVHD. To induce cGVHD, $1.2x10^6$ splenocytes from B6 donors were injected into lethally irradiated Balb/c recipients. These mice usually show clinical symptoms of cGVHD within 60 days and survival of mice at day 60 is around 20% (data not shown). Therefore, mice received KRP203 either prophylactically and continuously until day 60 or for 1 week only (day-1 until day 7). Importantly, WD of KRP203 administration for no longer than 1 week resulted ultimately in the same survival rate as mice that received continuous KRP203 (around 40%) (Figure 17E).

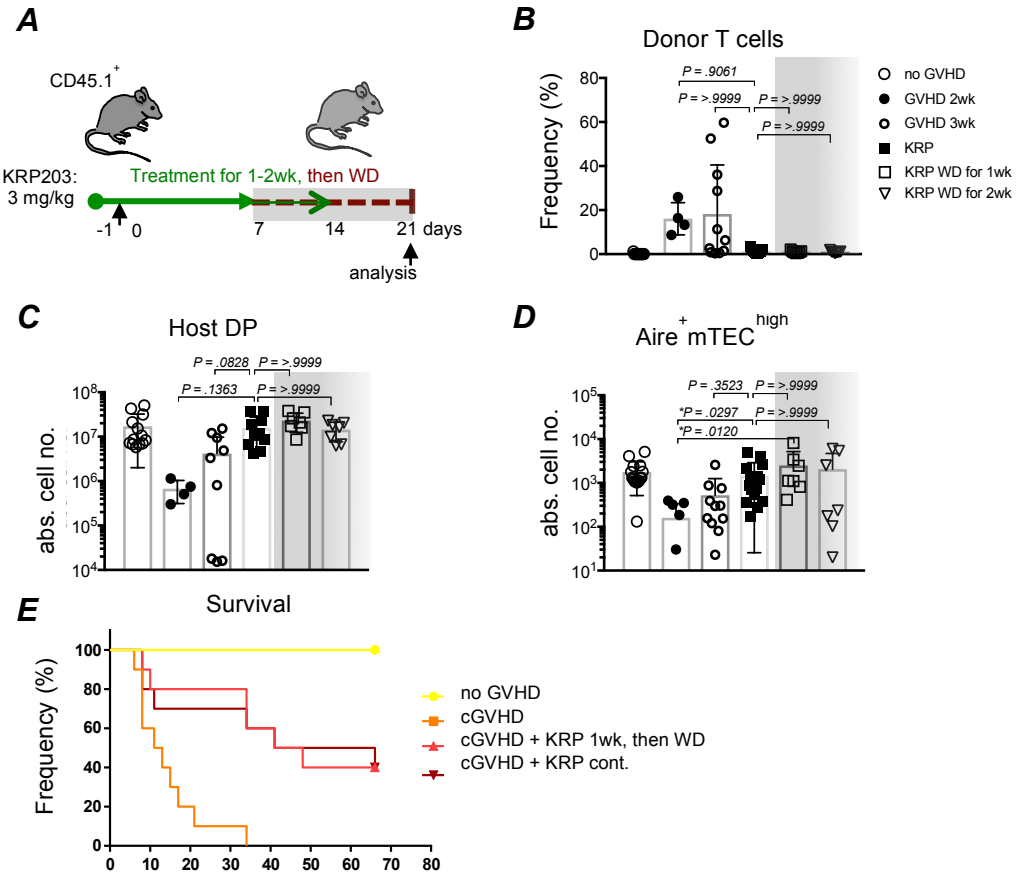


Figure 17: ***After withdrawal, the effect of KRP203 is maintained for a limited time period.*** *Acute GVHD (b→bd) was induced in 8-week old female BDF1 recipients by injection of splenic T cells from B6 donors. To investigate the effect after withdrawal (WD), mice received prophylactic KRP203 administration (day-1 until end of experiment) (■), KRP203 for 2 weeks and WD for 1 week (□) and KRP203 for 1 week and WD for 2 weeks (▽). As control, mice without KRP203 were analyzed with GVHD at 2 weeks (●), GVHD at 3 weeks (○) and no GVHD (O). Mice were analyzed 3 weeks after T-cell transfer (A). Frequencies of donor T-cell infiltration ($CD45.1^+$) into the thymus were determined (B). Absolute cell numbers of host DP were analyzed (C) and absolute cell numbers of $Aire^+mTEC^{high}$ cell*

*numbers were determined (D). The graphs represent pooled data from 3 independent experiments with ≥3 mice per group. *p< 0.05, Kruskal-Wallis and Dunn's multiple comparison test. Chronic GVHD (cGVHD) was induced by the transfer of low numbers of splenocytes into lethally irradiated Balb/c mice. The survival curve was generated over time and shows mice that did not develop GVHD (yellow line), mice that developed cGVHD (bright orange), mice with cGVHD but KRP203 for 1 week, then WD (dark orange) or mice with continuous KRP203 application (brown line). This graph is representative from data from Phil Smith (Novartis Inc.).*

From the combined data, I concluded that the beneficial effect of prophylactic KRP203 administration could be maintained even after WD (albeit a time span of only 1-2 weeks was tested). Moreover, the reduced number of alloreactive T cells invading the thymus might consequently have had a beneficial effect on the thymic stromal compartment – a direct target of alloreactive donor T cells during aGVHD.

5.3.4 KRP203 allows for normal intrathymic T-cell regeneration in transplanted recipients with aGVHD

Reconstitution of a functional T-cell pool in a fast and competent manner is critical for a successful HSCT outcome. However post-transplantation T-cell regeneration is impeded by aGVHD.[21] To investigate whether KRP203 administration could improve thymus-dependent T-cell regeneration in the presence of aGVHD, I took the same fully myeloablated MHC-mismatched model as described above (chapter 5.2.3) but co-transferred donor mature T cells (*Balb/c→B6CD45.1*$^+$ = H-2^d→H2^b; *d→b*). This aGVHD model is characterized by injury of the typical target organs, but also by impairment in thymic function and a delayed peripheral T-cell regeneration.[134] 2 weeks post allo-HSCT, mice were sacrificed, the thymus was analyzed and absolute cell numbers were counted. To discriminate between donor and host cells, I firstly gated on CD45.1$^+$ (host) and CD45.1$^-$ (donor) cells (Figure 18A, first column). I intentionally did not use H-2^b/H-2^d as markers to distinguish donor and host since MHC expression presents a continuum on early developing thymocytes. Among the CD45.1$^+$ host compartment, I investigated cell population frequencies by gating on CD4$^+$ and CD8$^+$ T cells. Among the CD45.1$^-$ compartment, I further distinguished between donor mature T cells (Thy1.2$^+$) and TCDBM-derived T cells (Thy1.2$^-$) that were contained in the graft (Figure 18A, middle column). I further differentiated between CD4$^+$ and CD8$^+$ T cells among Thy1.2$^+$ and Thy1.2$^-$ cells (Figure 18A, column 4 and 5 respectively). Based on the T-cell subset determination by flow cytometry, I calculated absolute cell numbers of donor-BM DP, DN, CD4SP and CD8SP populations. Mice that received TCDBM alone showed a normal T-cell regeneration, beginning with a normal development of DN to DP subsets, as expected at 2 weeks post-HSCT (Figure 18B). In the presence of aGVHD however, intrathymic T-cell development was delayed with regard to immature DP cell population sizes compared to the control group: The DN compartment was

increased in the presence of aGVHD in comparison to control mice ($4.9\pm2.4x10^5$ to $2.1\pm3.4x10^5$) while the DP compartment was decreased ($1.1\pm1x10^3$ to $2.4\pm4.7x10^5$) respectively. This aberrant intrathymic T-cell maturation process could be prevented by the prophylactic administration of KRP203 (DN compartment $1.2x10^5\pm7.7x10^4$, DP compartment $2.4\pm2.2x10^4$). Importantly, allogeneically transplanted mice that received KRP203 did not show any significant difference to control mice that received no alloreactive T cells (TCDBM alone) (Figure 18B). Hence, the presence of KRP203 allowed for normal thymus-dependent T-cell development most likely by preventing injury to elements of the host thymic microenvironment (see next chapter).

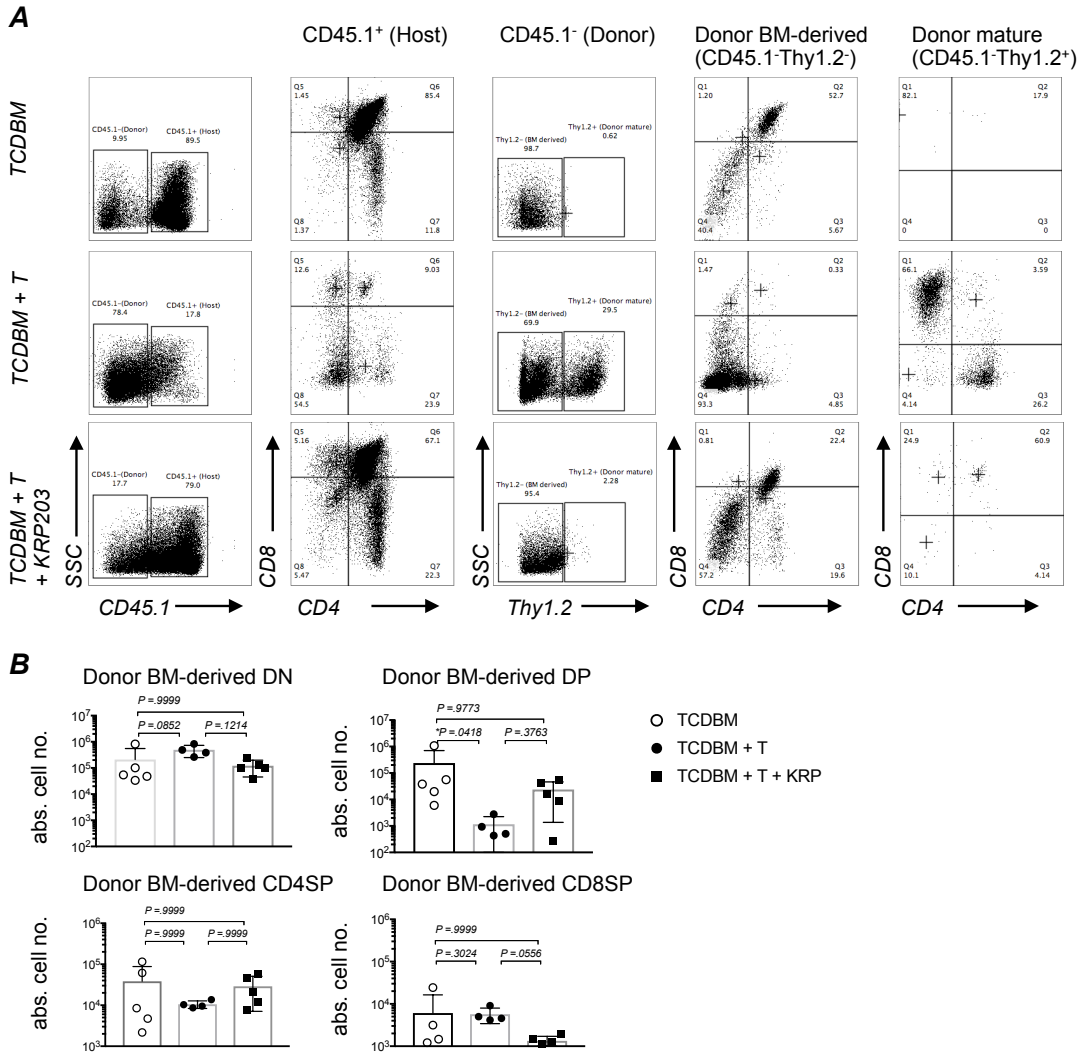


Figure 18: ***KRP203 allows for normal intrathymic T-cell regeneration in transplanted recipients with aGVHD.*** *In a fully MHC-mismatched murine allo-HSCT model (H-2^d→H-2^b) acute GVHD (aGVHD) was induced in lethally irradiated B6 recipients by co-injection of TCDBM + T cells (aGVHD (●)). The second group received TCDBM + T cells + KRP203 (3mg/kg, i.p., every 2nd day from day-1 until end of experiment) (■) and the control group received TCDBM alone (○) from Balb/c mice. Mice were sacrificed at 2 weeks post allo-HSCT. Thymic cellularity was analyzed by gating on the CD4 and CD8 compartment of the host thymus (CD45.1$^-$) (2nd column from left) and on the donor compartment (CD45.1$^+$) (3rd column from left). Among CD45.1$^+$ cells, donor BM-derived (Thy1.1$^+$) T cells can be*

*separated from donor mature T cells (Thy1.2$^+$) (4th and 5t^h column from left respectively) as it is demonstrated in representative FACS plots (A). Absolute cell numbers of donor BM-derived DP, DN, CD4SP and CD8SP T cells were determined (B). The graphs represent data from one experiment with n=5 mice per group. *p< 0.05, Kruskal-Wallis and Dunn's multiple comparison test.*

5.4 Cytoprotection by KRP203 of mTEC prevents the release of *de novo* generated autoreactive T cells

5.4.1 KRP203 protects the thymic mTEC compartment

Our laboratory has demonstrated earlier that alloantigen-specific recognition of host TEC by mature donor T cells provides the principal injury that limits thymocyte expansion and selection in mice with aGVHD.[134] To investigate the direct consequence of reduced numbers of intrathymic alloreactive donor T cells upon KRP203 administration on the thymic microenvironment, I used the same mouse model as described above (*B6→BDF$_1$* = H-2^b → H-2^{bd}; *b→bd*) (see chapter 5.3.1). 2 weeks after T-cell transfer, the mTEC compartment was analyzed by flow cytometry using the following markers: CD45$^-$EpCAM$^+$Ly51$^-$UEA-1$^+$MHCII^{high} (Figure 19A). As expected and described before,[129] mice with aGVHD had significant lower numbers of mTEC^{high} in comparison to control mice (5.9±4.7x10^3 to 1.2±1x10^4 respectively, **P=0.0293*) (Figure 19B). Mice that received KRP203 had absolute mTEC numbers that were comparable to control mice (*P=0.8120*) which were higher than in mice with aGVHD (7.1±5.4x10^3, **P=0.0497*) (Figure 19B, upper panel). Importantly, numbers of mTEC^{high} expressing the TF *Aire* in mice that received KRP203 were comparable to control mice with respect to absolute cell numbers (*P=0.0680*) and significantly higher than in mice with aGVHD (from 1±1.3x10^3 to 2±1.7x10^3, ***P=0.0020*) (Figure 19B, lower panel). I further confirmed these data with immunofluorescence microscopy where I examined the expression of *Aire* (PE Cy5, red) by CK14$^+$ mTECs (A488, green) (Figure 19C). The results observed by flow cytometry were substantiated in two other irradiated allo-HSCT models that included a fully myeloablated MHC-mismatched model (Balb/c→B6 = H-2^d→H-2^b; *d→b*) at 2 weeks post allo-HSCT (Figure 19D, upper graph) and a MHC-matched but minor HA-mismatched cell transfer (*B6129S1/Sv* → B6Ly5.1; *b→b*). The latter model also has defective thymopoiesis as hallmark of aGVHD but defects are not as strong as in the MHC-mismatch model. Therefore, I analyzed these mice at 4 weeks post allo-HSCT. In both models, Aire$^+$mTEC^{high} numbers were determined. Consistent with previous results, I observed a trend towards increased Aire$^+$mTEC^{high} numbers in the presence of KRP203 (Figure 19D, lower graph).

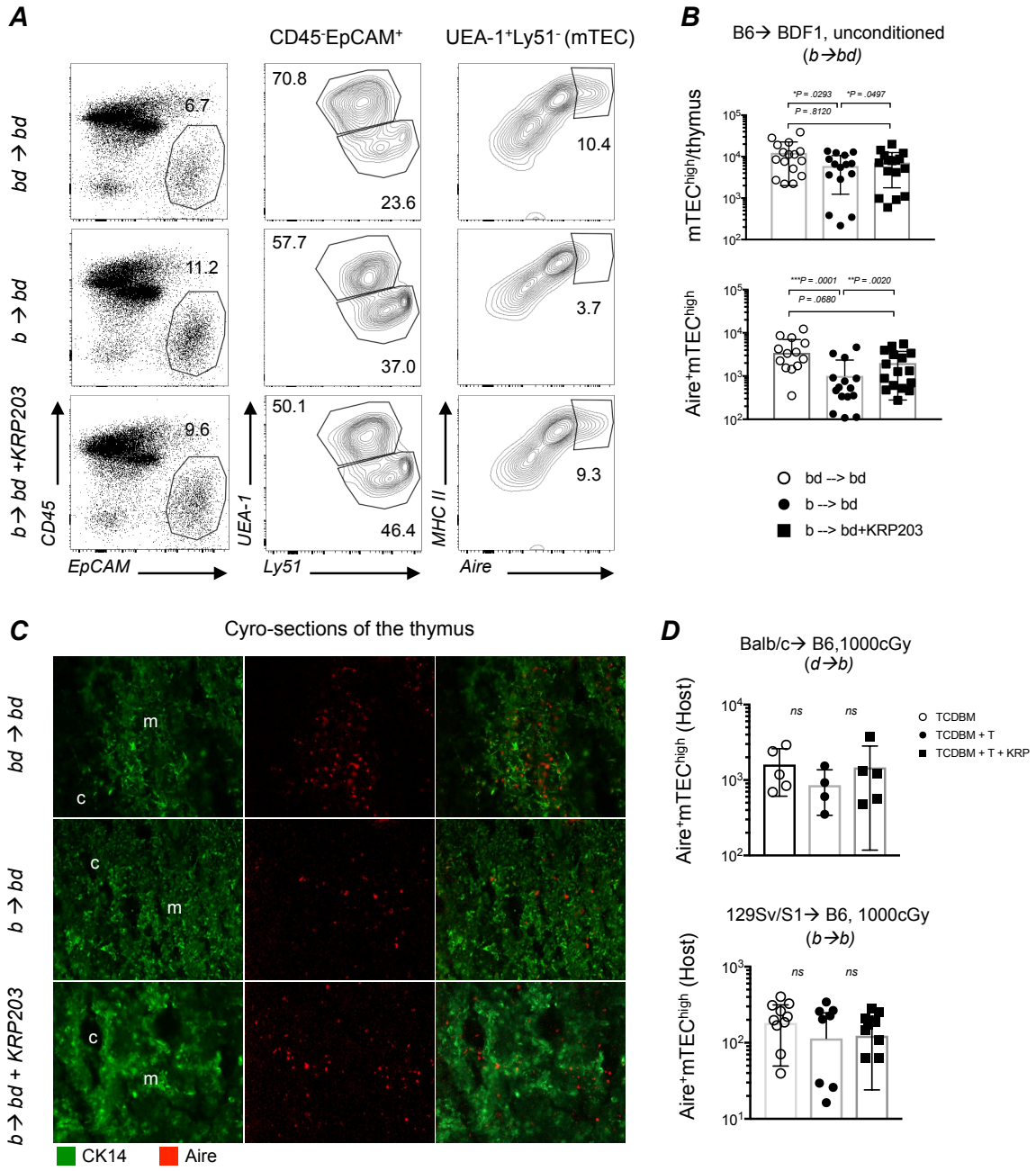


Figure 19: ***KRP203 prevents damage to the thymic epithelial microenvironment during aGVHD.*** *Acute GVHD (aGVHD) was induced in 8-week old female BDF1 recipients by injection of splenic T cells from B6 donors without KRP203 (b→bd) or with KRP203 (b→bd+KRP203). As controls, mice without aGVHD received T cells from a syngeneic donor (bd→bd). The gating strategy is displayed as FACS plots from one representative experiment. Thymic epithelial cells (TEC) express the surface marker EpCAM but are negative for CD45 (left column). Medullary TEC (mTEC) and cortical TEC (cTEC) can be differentiated with UEA-1^{+}Ly51^{-} and Ly51^{+}UEA-1^{-} respectively (middle column). Mature mTEC further express MHCII^{high} and Aire (right column) (A). Absolute cell numbers of mTEC^{high} and Aire^{+}mTEC^{high} cells were examined from mice without aGVHD (○), mice that developed aGVHD (●) and mice with aGVHD and KRP203 (3mg/kg, i.p., ever 2nd day from day-1 until the end of experiment) (■) (B). The graphs represent data from 4 independent experiments with ≤ 4 mice per group. Immunofluorescent microscopy was performed from frozen thymus sections. The medulla was stained with Cytokeratin 14 (CK14, green) and Aire (red). The cryo-sections*

are shown from one representative mouse of each group (C). Absolute cell numbers of Aire$^+$mTEC^{high} cells were determined from a lethally irradiated MHC-mismatched model (d→b) ((D) upper graph)) at 2 weeks post allo-HSCT and minorHA-mismatch (b→b) at 4 weeks (lower graph) ((D, lower graph). p< 0.05, two-way ANOVA and uncorrected Fisher's lsd test.*

From the combined data I concluded that prophylactic and continuous administration of KRP203 allowed - indeed via prevention of donor T-cell passage into the thymus - to maintain a normal thymus stromal microenvironment and hence should allow for a normal thymopoiesis to occur following allo-HSCT. This in turn might sustain a normal negative selection process during aGVHD, a hypothesis that was tested in the next chapters.

5.4.2 KRP203 allows for a normal negative selection process in thymus

The exposure of developing T cells to self-antigens expressed in the thymus is essential for clonal deletion. Strongly self-reactive T cells undergoing negative selection induce Helios and PD-1 expression[101,102] in response to self-antigens that are particularly expressed by the Aire$^+$mTEC^{high} compartment. During aGVHD, injury to the stromal network, including Aire$^+$mTEC^{high}, disables these cells to serve as a healthy platform for central T-cell tolerance induction.[129] The preserved Aire$^+$mTEC^{high} pool size in allo-HSCT recipients receiving alloreactive T cells together with prophylactic KRP203 was promising especially in light of the potential of maintaining a normal negative selection despite aGVHD. I consequently tested whether KRP203 administration would improve thymic negative selection following allo-HSCT. Therefore, I used the haplo-ID non-conditioned model as described before (*B6→BDF$_1$* = H-2^b → H-2^{bd}; *b→bd*) (see chapter 5.3.1). At 3 weeks after T-cell transfer, mice were sacrificed and T-cell subsets were analyzed by flow cytometry. In order to detect negative selection marker, I gated on either host DN or CD4SP cells. Among these subsets, I further gated on TCRβ$^+$CD5$^+$CCR7$^-$ cells. Negatively selected cells could further be characterized trough their PD-1 and Helios induction (among DN) or Helios induction alone (among CD4SP) (Figure 20A). Mice that did not develop aGVHD, had 40±18% PD-1$^+$Helios$^+$ cells among the CD4$^-$CD8$^-$CD5$^+$CCR7$^-$ population. In contrast, mice that did develop aGVHD had much lower frequencies of PD1$^+$Helios$^+$ cells or Helios$^+$ cells (18.8%), indicating that during aGVHD, thymocytes could not induce these markers anymore in order to become negatively selected. Mice that received prophylactic KRP203 administration had comparable frequencies (40±10%) of CD4$^-$CD8$^-$TCRβ$^+$CD5$^+$CCR7$^-$ PD-1$^+$Helios$^+$ cells to control mice (Figure 20B). Among the CD4$^+$CD8$^-$TCRβ$^+$CD5$^+$CCR7$^-$ population, control mice had 3.2% of Helios$^+$ cells. In the presence of GVHD, mice had <2% Helios$^+$ cells whereas mice that received KRP203 showed even higher frequencies (3.6%) of Helios-inducing cells (Figure 20C).

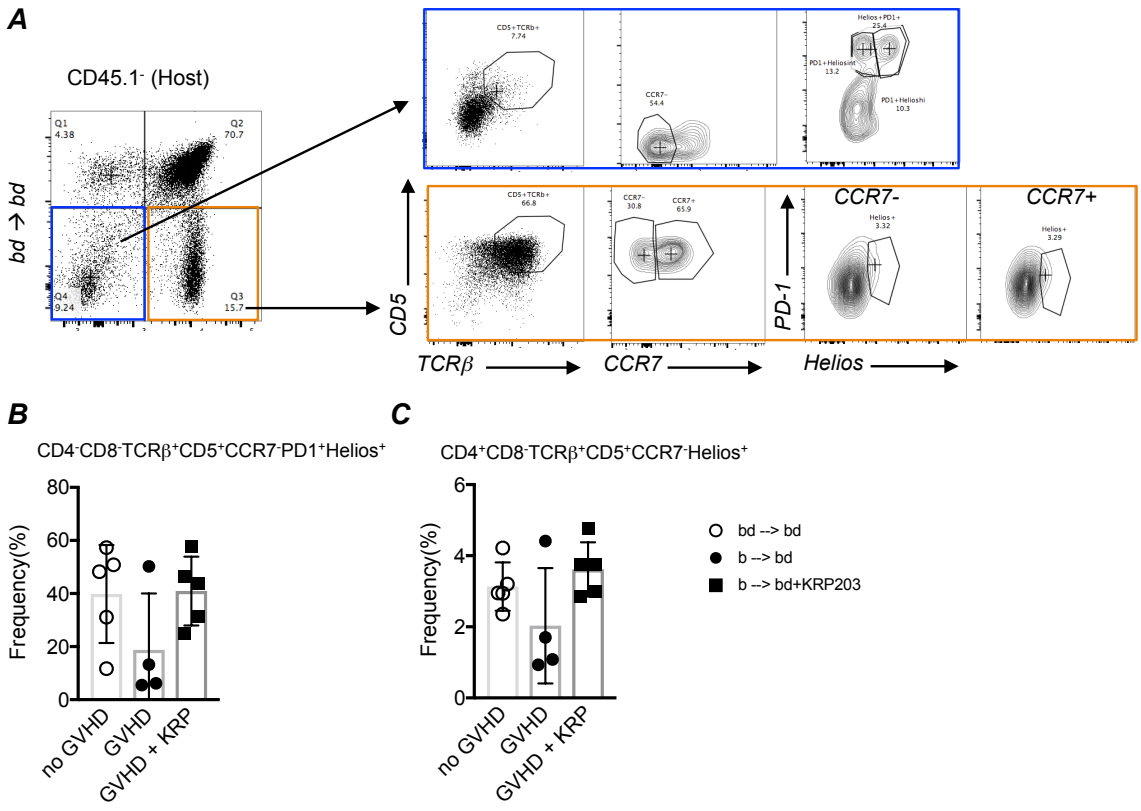


Figure 20: ***Negative selection marker Helios cannot be induced in aGVHD but rescued with KRP203 application.*** *Acute GVHD (aGVHD) (b→bd) was induced in 8-week old female BDF1 recipients by injection of splenic T cells from B6 donors for 3 weeks. The gating strategy is displayed as FACS plot from one representative experiment. Negatively selected T cells can be detected among the DN compartment or among CD4SP cells. Thymocytes that undergo negative selection express TCRβ and are negative for CD5. Among TCRβ^{+}CD5^{-} they are negative for CCR7 while PD-1 and Helios or Helios expression alone is induced among DN or CD4SP respectively (A). Absolute cell numbers were determined of cells that were marked to become negatively selected and express PD1 and Helios (B) or Helios alone (C). Data are shown from one representative experiment including mice without aGVHD (○), mice that developed aGVHD (●) and mice with aGVHD and KRP203 administration (3mg/kg, i.p., every 2nd day from day-1 until the end of experiment) (■). The graphs demonstrate representative data from one experiment with n=5 mice per group. *p< 0.05, Kruskal-Wallis and Dunn's multiple comparison test.*

In summary, my data indicated that self-reactive T cells were not able to induce Helios or PD-1 anymore in the presence of aGVHD. These markers are crucial for strongly self-reactive T cells to become negatively selected. Thus, a damaged Aire^{+}mTEC^{high} compartment might directly link donor T-cell alloimmunity to the appearance of autoreactive T-cell clones that may, however be prevented by KRP203 administration.

5.4.3 KRP203 reduces the release of *de novo* generated autoreactive T cells during aGVHD

As the above results demonstrated, it is the protection of the mTEC pool that governs the efficacy of thymic negative T-cell selection as it regulates the expression levels of self-antigens. Importantly, intimate associations exist between perturbations in TRA expression and the susceptibility to autoimmunity. This led me further to the study whether preserved central tolerance via KRP203 may hence prevent the emergence of autoreactive T cells. To this end, I used an established transgenic model using B6 RIP-mOVA mice (Figure 21A) that express mOVA as a surrogate self-antigen in the medulla of the thymus[201,203] (see chapter 5.1). First, I wanted to investigate if the $Aire^{+}mTEC^{high}$ pool can be preserved in a fully myeloablated MHC-mismatched model (*Balb/c→B6* = H-2^{d}→H-2^{b}; *d→b*) at 4 weeks post allo-HSCT. This model was comparable to the transgenic Balb/c→RIPmOVA model with respect to impaired thymopoiesis and defect in the $Aire^{+}mTEC^{high}$ compartment during aGVHD. At 4 weeks post allo-HSCT, absolute $Aire^{+}mTEC^{high}$ numbers were determined. Control mice received TCDBM alone showed normal numbers ($7.4\pm5.5x10^{3}$) of $Aire^{+}mTEC^{high}$ in this conditioned mouse model (Figure 21B). Mice that developed aGVHD after co-injection of TCDBM+ T cells had diminished $Aire^{+}mTEC^{high}$ numbers ($2.5\pm3.4x10^{3}$) whereas mice that received TCDBM + T cells + KRP203 showed higher numbers than untreated mice with aGVHD ($3.8\pm4.3x10^{3}$, *P=0.9764*), albeit not significantly different (Figure 21B). The next step was to induce GVHD in the transgenic model. Therefore, RIPmOVA mice were lethally irradiated and transplanted with $7x10^{6}$ TCDBM from Balb/cThy1.1^{+} (no GVHD) or $7x10^{6}$ TCDBM + $1x10^{6}$ T cells from Balb/cThy1.2^{+} (aGVHD) and were either left untreated or treated with KRP203 administration for 4 weeks. At 4 weeks after the 1st allo-HSCT, mice were lethally re-irradiated and transplanted with TCDBM from B6 and B6OT-II^{+}CD45.1^{+} mice (4:1 ratio). OT-II^{+} BM derived cells contained a TCR ($V\alpha2^{+}V\beta5^{+}$) specific for OVA peptide and could therefore be detected by flow cytometry (Figure 21C). Another 4 weeks later, mice were sacrificed and OT-II-specific T-cell numbers were determined in the peripheral LN. In the TCDBM group, only low numbers of $CD4^{+}CD45.1^{+}$ T cells were detected in the peripheral LN ($1.3\pm1.5x10^{4}$) (Figure 21D). Here, BM-derived cells T cells underwent thymus-dependent negative selection, as expected, and OT-II^{+} cells were negatively selected and eliminated. In mice that had developed aGVHD $CD4^{+}CD45.1^{+}$ T cells were, however, present in higher numbers ($1.3\pm1.5x10^{5}$). This observation strongly indicated that inadequate clonal deletion of OVA-reactive T cells resulted from loss of thymic OVA expression during aGVHD (see also Appendix, Manuscript Figure 1). Hence, OVA-specific cells could escape negative selection and were released into the periphery as *de novo* generated autoreactive T cells. As a control, non-tg B6 recipients received TCDBM from B6 and B6OT-IICD45.1^{+} mice. Here, the OT-II specific TCR did not recognize OVA peptide and none of the

OT-II$^+$ cells were deleted in the thymus. As a consequence, high numbers of CD4$^+$CD45.1$^+$ T cells were present in the periphery (5.9 x10^6±1x10^7). In contrast to mice with aGVHD, mice that received KRP203 within the first 4 weeks had a diminished number of autoreactive T cells (2.8±3.6x10^4, *P=0.4235*) present in the periphery (Figure 21D). To specify the CD45.1$^+$ subset, I further gated on the OT-II specific TCR clone chains Vα2$^+$Vβ5$^+$. Confirming the above results, the group that received TCDBM only had no CD4$^+$CD45.1$^+$Vα2$^+$Vβ5$^+$ T-cell numbers present in the periphery. Mice that developed aGVHD within the first 4 weeks had higher numbers of CD4$^+$CD45.1$^+$Vα2$^+$Vβ5$^+$ T cells (1.3±3x10^4), non-tg B6 mice had even higher numbers (6.5x10^5±1x10^6) whereas mice with KRP203 had low numbers of CD4$^+$CD45.1$^+$Vα2$^+$Vβ5$^+$ T cells (5.6x10^2±1x10^3) (Figure 21E).

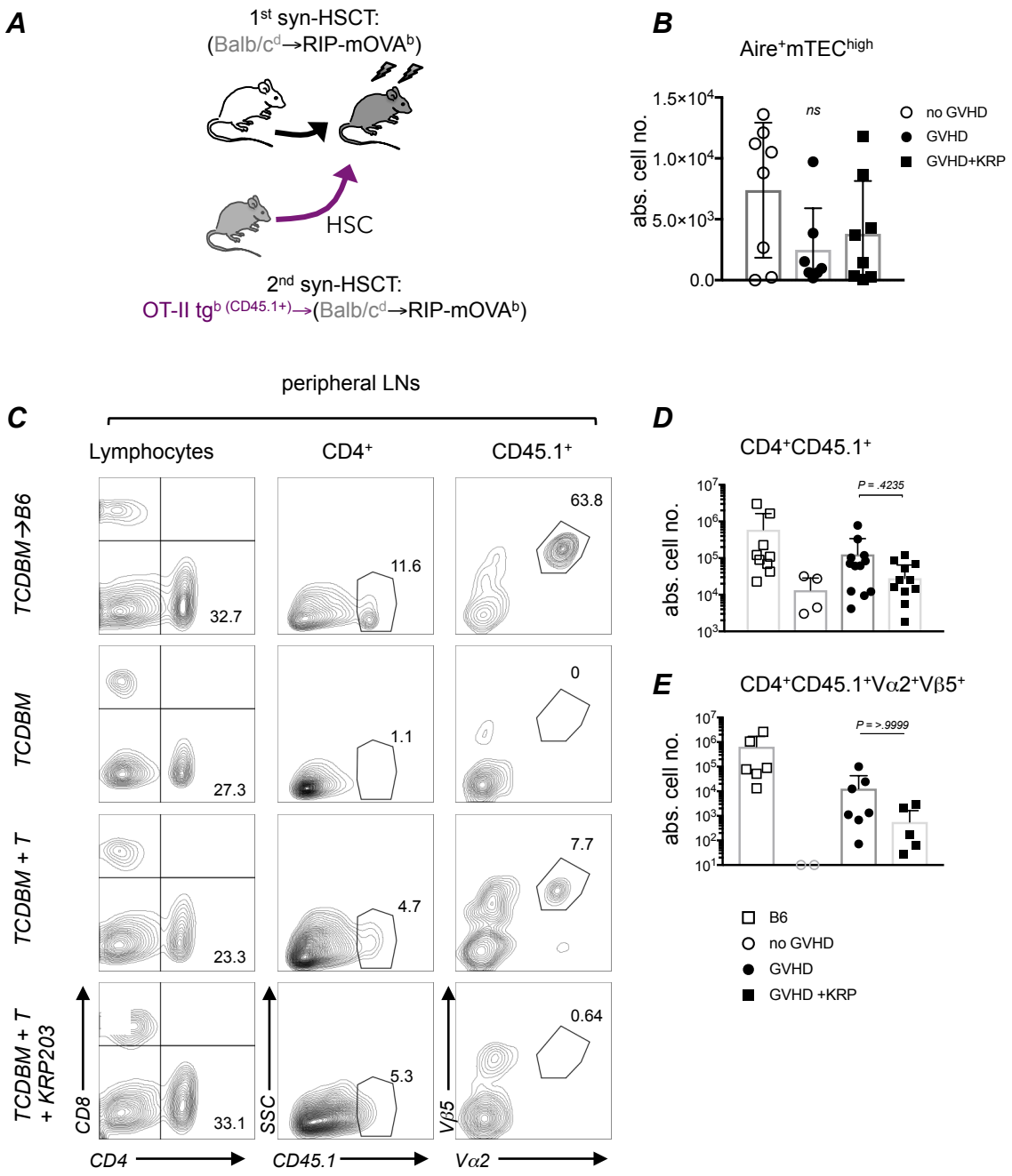


Figure 21: ***KRP203 reduces the release of de novo generated autoreactive T cells during aGVHD.*** *Mouse model to study central tolerance during acute GVHD (aGVHD) as described in Figure 8 (A). Absolute cell numbers of Aire^{+}mTEC^{high} cells were calculated from the host thymus in lethally irradiated B6 mice that received TCDBM only (○), TCDBM + T cells (●) and TCDBM + T cells + KRP203 (■) from MHC-mismatched Balb/c donors (H-2^{d}→H-2^{b}) at 4 weeks post allo-HSCT (B). FACS plots from one representative experiment are depicted for the gating strategy of ovalbumin (OVA)-specific T cells in the peripheral lymph nodes (pLN). De novo generated OVA-specific CD4^{+} T cells express the congenic marker CD45.1^{+} (left and middle column) and the OTII specific TCR chain Vα2^{+}Vβ5^{+} (right column) (C). Absolute cell numbers of CD4^{+}CD45.1^{+} OTII-specific T cells were determined from mice that received TCDBM only (○), mice that received TCDBM + T cells (●), mice that received TCDBM + T cells + KRP203 during 4 weeks after the first allo-HSCT (■) and B6 mice as positive control (□) (D). The graph represents data from 5*

*combined experiments. Absolute cell numbers of TCR clones expressing the Vα2$^+$Vβ5$^+$ chain among CD4$^+$CD45$^+$ cells were determined in the pLN (E). The graph depicts values from 4 different experiments. KRP203 application was the same for all mice (3mg/kg, i.p., ever 2nd day from day-1 until the end of experiment). *p< 0.05, Kruskal-Wallis and Dunn's multiple comparison test.*

These data indicated that elimination of mTEC and hence failure to express OVA in the thymus, led to the emergence of *de novo* generated OVA-specific autoreactive T-cell clones as a consequence of thymic aGVHD. This deficit, however, could be prevented to a large degree by the presence of KRP203.

5.5 Graft-vs- Tumor activity is maintained under the KRP203 umbrella

5.5.1 T cells accumulate in SLO under KRP203 treatment

The data shown above illustrated that alloreactive donor T cells did not infiltrate the thymus upon KRP203 administration even in the presence of conditioning-related toxicity. Thus, the absence of donor T cells in the thymus directly led to the next question whether donor mature T cells were accumulating in SLO. Therefore, I took the same fully myeloablated MHC-mismatched model as described above (chapter 5.3.4) (*Balb/c→B6CD45.1*$^+$ = H-2^d→H2^b; *d→b*) where spleen and LN were analyzed at 2 weeks post allo-HSCT. Donor mature T cells were identified by the surface expression of H-2d$^+$ cells (Figure 22A, 2nd column). At 2 weeks post-HSCT, only few thymus-derived T cells were yet expected to be detectable in the periphery, as confirmed by low absolute cell numbers in the control group that received TCDBM only (upper row). Therefore, no further marker was necessary to distinguish between donor mature T cells and donor BM-derived T cells. Among H-2d$^+$ cells, I further gated on CD4 and CD8 (Figure 22A, 3rd column). Based on this gating strategy, I calculated absolute cell numbers for both, spleen and LN. In the spleen, TCDBM control mice showed low numbers of CD4$^+$ donor T cells (1.8±1x10^4) whereas in mice receiving T cells or T cells+KRP203, CD4$^+$ T-cell numbers were >1log higher (2.5±1.5x10^5 and 1.7±1x10^5 respectively). The same was true for CD8$^+$ T cells which were higher in mice with aGVHD and aGVHD+KRP203 (6.3±3.4x10^5 and 3±2.1x10^5 respectively) in contrast to control mice that received TCDBM only (5.5±3x10^4) (Figure 22B). I could substantiate this observation in the peripheral LN. Mice that received TCDBM alone had low numbers of CD4$^+$ donor T cells (1.6±2.2x10^3), whereas mice that received additional T cells and T cells + KRP203 showed increased numbers of donor T cells within the LN (1.2x10^4±8.9x10^3 and 3.5±3.1x10^3 respectively). However, I could not detect high numbers of CD8$^+$ donor T cells when mice received KRP203 (6.2±6x10^3) in contrast to TCDBM (6.8±8.2x10^3) and TCDBM + T cells (1.5±1.2x10^4) (Figure 22C).

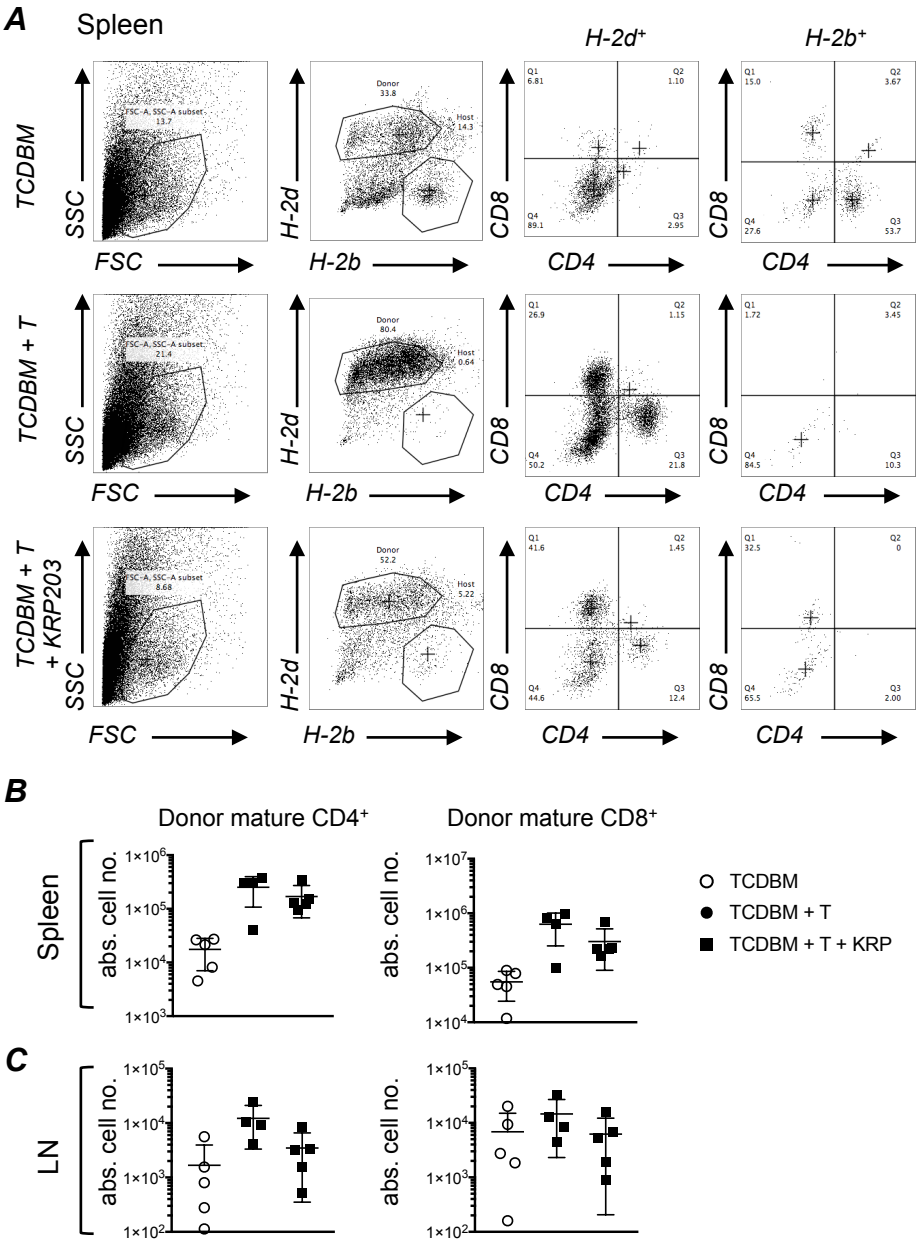


Figure 22: ***Donor mature T cells are "trapped" in secondary lymphoid organs upon KRP203 administration in the presence of aGVHD.*** *In a fully MHC-mismatched murine allo-HSCT model (H-2^d→H-2^b) acute GVHD (aGVHD) was induced in lethally irradiated B6 recipients by co-injection of TCDBM + T cells (aGVHD (●)) TCDBM + T cells + KRP203 (3mg/kg, i.p., every 2nd day from day-1 until the end of experiment (■)) or TCDBM alone (○) from Balb/c mice. Mice were sacrificed 2 weeks post allo-HSCT and spleen and lymph nodes (LN) were analyzed. Representative FACS plots of one mouse per group depicts the gating strategy. Among all lymphocytes (1st column) one can differentiate between donor (H-2d$^+$) and host (H-2b$^+$) T cells (3rd and 4th column respectively) (A). Absolute cell numbers of CD4$^+$ and CD8$^+$ T cells were determined in the spleen (B) and LN (C). The graphs represent data from one experiment with n=5 mice per group.*

Taken together, the data hence demonstrated that prophylactic and continuous KRP203 administration could prevent an aGVHD-mediated or conditioning-related injurious effect on thymus-dependent T-cell regeneration with regard to cell pool sizes. Concomitantly, in mice with

KRP203 there was an accumulation of donor mature T cells within SLO at 2 weeks post-HSCT. This observation was highly relevant and strongly implied a functional effect of donor mature T cells on tumor cells residing within SLO.

5.5.2 GVT effect is maintained under the KRP203 umbrella

Donor mature T cells, rather than HSC-derived thymus-dependent T cells, may represent the major therapeutic principle to cure patients from malignant hematological disease. Although previous studies have demonstrated that GVT activity is mostly retained under FTY720 administration, I did not know whether the same applied for KRP203, a drug with a narrower binding specificity with S1P receptors. The data above raised thus the possibility that the GVT effect might still have been preserved due to the fact that donor mature T cells indeed were "trapped" within SLO post allo-HSCT. Before I could investigate the GVT effect in a transplantation setting, I had to establish a lymphoma tumor model. A lymphoma tumor might be good target of alloreactive donor T cells under the KRP203 umbrella because the tumor resides in SLO where donor T cells were observed to be trapped. I took the A20 B-cell lymphoma cell line, generated in Balb/c (H-2^d) mice containing a luciferase reporter protein.[212] This A20 reporter cell line can be tracked *in vivo* by bioluminescence imaging, making it a great tool to study tumor growth. To test tumor development, I injected $1x10^4$ A20 cells directly into the left inguinal LN and obtained bioluminescence images 4 days post-injection (Figure 23A). At day 4, the mouse was sacrificed (Figure 23B) and I compared absolute cell numbers of the right non-injected LN versus the left LN containing A20 cells ($<5x10^6$ to $>1.5x10^7$ respectively) (Figure 23C).

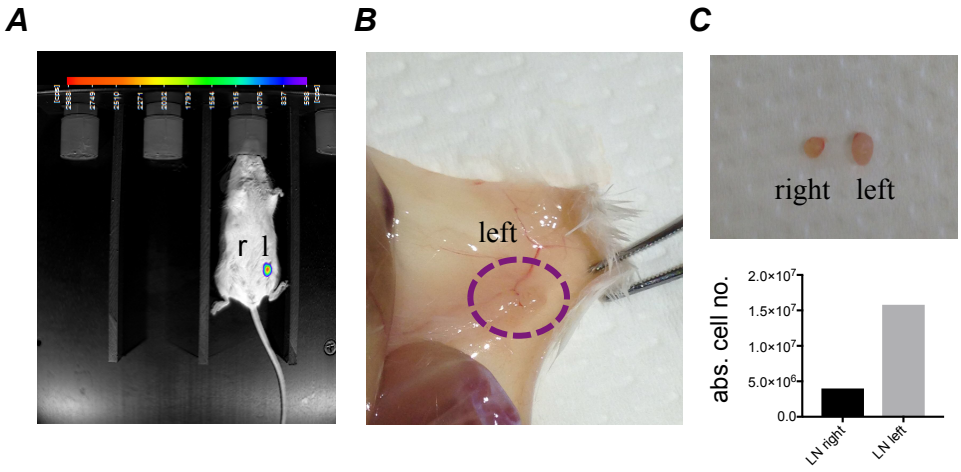


Figure 23: ***Establishment of a lymphoma tumor model****. 1x10^4 A20 luc$^+$ cells were directly injected into the left-side inguinal LN of a WT B6 mouse. Bioluminescence image of a mouse 4 days after tumor injection into the left LN (A). Left-side inguinal LN 7 days after tumor injection (B). The picture shows the comparison of the size of the right LN without injection and left LN with injected cells. Absolute cell numbers of both LN were counted (C).*

Based on the image, size of the LN and cumulative cell numbers of the left inguinal LN, I decided to use this method as a forthcoming tumor model in a transplantation setting. To address the question if KRP203 still provided the GVT effect while preventing aGVHD, I lethally irradiated Balb/c recipients and transplanted them with 7x10^6 TCDBM from B6 donors or 7x10^6 TCDBM + 2x10^6 T cells (*b→d*) with or without KRP203. One day after allo-HSCT, mice were injected directly into the left inguinal lymph node with 1x10^4 luciferase$^+$A20 lymphoma cells. The tumor development was observed by bioluminescence imaging 2.5 and 3.5 weeks after HSCT+A20 injection. Mice that received TCDBM alone developed lymphoma. In contrast, mice that received TCDBM+T cells were able to attack and eliminate the tumor. The latter was also true for mice that received TCDBM+T cells + KRP203 (Figure 24A). Mice that received TCDBM alone showed a tumor size in the range of 120-150mm^2. In contrast, mice with TCDBM+T cells +KRP203 had a tumor size in the range of 0-30mm^2. Mice that received TCDBM + T cells did not show any tumor development, as expected. The signal intensity of TCDBM recipients was in the range of ~50x10^6 photons/sec whereas the tumor of mice with KRP203 administration showed only low signal intensity in the range of 0.1-0.4x10^6 photons/sec (Figure 24B).

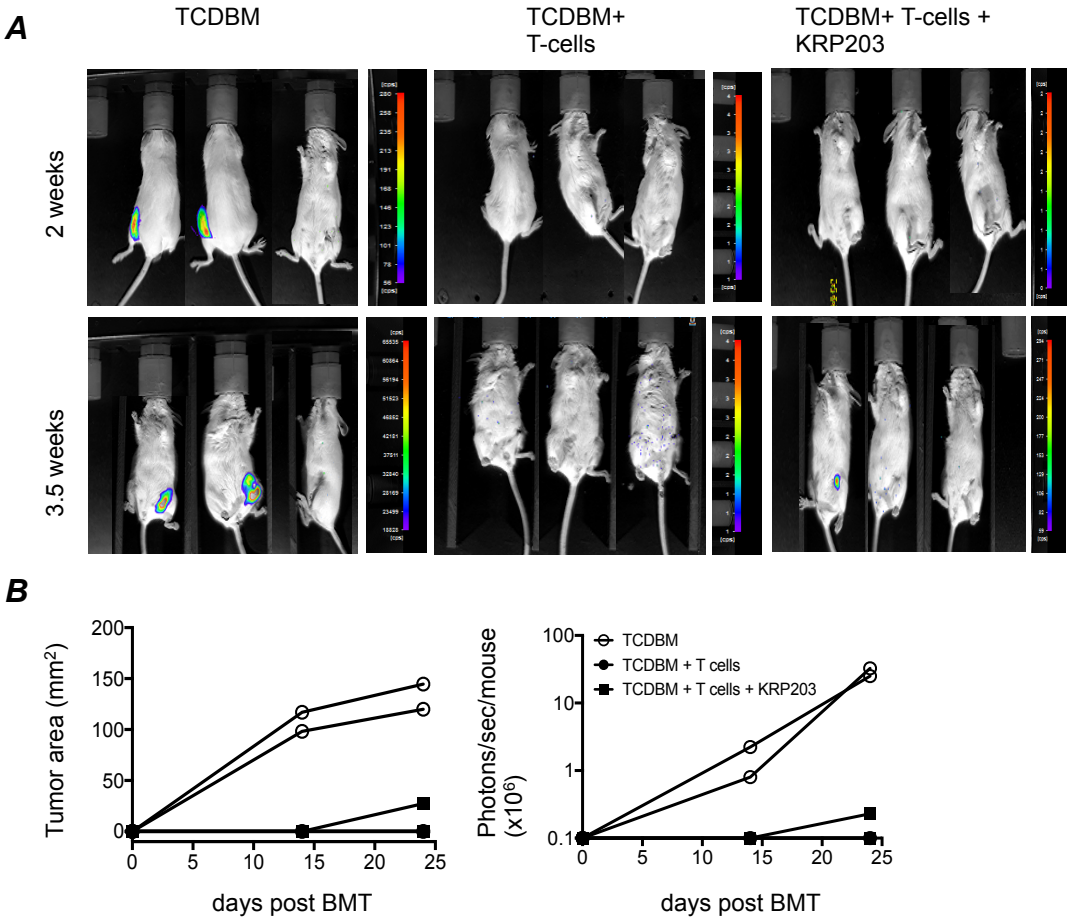


Figure 24: ***KRP203 preserves anti-tumor immunity****. The A20 lymphoma cell line was directly injected into the left inguinal LN (1x10^4 cells). The tumor cell line was positive for the luciferase and could be detected with D-luciferin injection 3min before imaging. One representative bioluminescence image is shown of each group of mice at 2 and 3.5 weeks post allo-HSCT; Mice that received TCDBM alone are shown in the left panel. Mice that received TCDBM + T cells are shown in the middle panel and mice that received TCDBM+ T cells + KRP203 are shown in the right panel (A). Tumor area and signal intensity of the A20 luc$^+$ lymphoma cell line were determined by the software of the NightOwl (B). Data are shown from one out of three independent experiments.*

Taken together, these data confirmed that induction of immune responses in lymphoid organs were preserved and thus maintained the capacity to reject hematopoietic tumors that are retained in these sites. An overview of the effect of KRP203 on all different mouse models tested is given in table 2 in the appendix.

6. Discussion

To restore immunity following allo-HSCT, an efficient reconstitution of the peripheral T-cell pool is essential and relies on a *de novo* generation of T cells in the recipient's thymus. However, the thymus has been identified as a target of TRT stemming from the conditioning regimen and from aGVHD which is a debilitating and often life-threating condition following allo-HSCT.[78] This toxicity hence negatively affects T-cell regeneration giving rise to posttransplantation immune deficiency, and possibly autoimmunity. cGVHD was originally regarded to be a continuation of aGVHD, primarily through expansion and persistence of donor T cells specific for MHC antigens or miHA uniquely expressed by recipient cells.[26,37,43] As an alternative explanation it was later considered possible that the thymus may play a role in the devlopment of cGVHD. An incomplete understanding regarding the molecular and cellular mechanisms underlying post-HSCT loss of central T-cell tolerance induction in the thymus provided the rationale for the first project. In collaboration with the first author of the paper, published in Blood 2015,[138] I was able to show in murine aGVHD models that autoreactive T cells can escape negative selection in a damaged host thymus during aGVHD and thus *de novo* generated autoreactive T cells can be exported to the periphery (see Appendix, Manuscript Figure 2A). With these data I could provide an important mechanistic link between the pathogenesis of aGVHD and cGVHD, which will be discussed in more detail in chapter 6.1. These results highly suggested for thymic cytoprotection via inhibiting T-cell migration into the thymus.

$S1PR_1$ agonism may successfully prevent aGVHD by inhibiting lymphocyte traffic from LN into peripheral tissues. Thus, I investigated how the $S1PR_1$-mediated receptor agonist KRP203 interfered with other important events that govern in combination the transplantation outcome: thymus-dependent T-cell regeneration, the preservation of the thymic microenvironment and consequent development of autoimmunity and anti-tumor immunity in allo-HSCT recipients. None of these variables could be addressed directly in the parallel clinical study in Basel. I could indeed demonstrate, firstly, in murine allo-HSCT models that KRP203 administration allowed for a normal intrathymic T-cell development post allo-HSCT (Figure 12). Secondly, I could show that KRP203 prophylaxis reduced alloreactive donor T-cell infiltration into the thymus (Figure 15). Consequently, detrimental effects of aGVHD on central tolerance induction (i.e. intrathymic deletion of *de novo* generated autoreactive T cells) could be prevented (Figure 21). Importantly, prophylactic KRP203 administration preserved anti-tumor immunity in an A20 mouse lymphoma model (Figure 24). This study will be discussed in chapter 6.2.

6.1 GVHD clears the Aire in thymic selection

During murine aGVHD, mTEC^{high} are targets of donor T-cell alloimmunity,[3,7,13] and thymic aGVHD interferes particularly with the capacity of Aire^{+}mTEC^{high} to sustain TRA diversity.[14] Mechanistic links between altered thymic TRA expression, and hence deviations in the TRA repertoire, the thymic production of autoreactive T cells, and ultimately their peripheral appearance during aGVHD (ultimately leading to cGVHD) have not yet been established. Unfortunately, the specificities of these autoreactive T cells present during cGVHD have not been identified to date and can thus not used to demonstrate direct evidence for a causal relationship with antecedent impaired TRA expression in the thymus. Therfore, I took advantage of the OT-II→RIP-mOVA mousel model, where OVA acts as a surrogate self-antigen.[201,203] This model is suitable to study thymic negative selection mediated by mTEC since OVA is expressed in their cell membrane under the control of the RIP-promoter, whose activity is restricted to pancreatic islets but also to Aire^{+}mTEC^{high} cells in the thymus. Thus, OVA expression acts as a tissue-specific protein that should cause negative selection in the thymus and normally should lead to elimination of autoreactive T cells that recognize mOVA peptides. To overcome the limitation that the unphysiological high frequency of OVA-specific precursors interferes with negative selection post allo-HSCT process,[206] I co-transferred OT-II TCDBM with TCDBM from WT B6 mice. This strategy ensures proper negative selection of OVA-specific T cells mimicking a normal scenario as it would be expected in normal T-cell development.

By using the transgenic RIP-mOVA mice as transplant recipients, I could show that mice without aGVHD efficiently deleted OT-II specific T cells as there were only low frequencies of OT-II^{+} T cells among all CD4^{+} T cells present in the peripheral LN and spleen. On the contrary, in non-transgenic WT B6 mice, which do not express mOVA, OVA-specific T cells were not negatively selected and thus could be released into the periphery. The induction of aGVHD in RIP-mOVA recipients, however, allowed the emergence of OT-II T cells to the periphery due to diminished thymic OVA expression. Indeed, aGVHD led to deletion of mTEC^{high} cells since the absolute cell numbers were decreased (Manuscript, Figure 1B). In contrast, Aire mRNA expression was not significantly affected by disease (Manuscript, Figure 1D). The question why aGVHD only interferes with Aire protein expression, but not on mRNA levels remains to be investigated. Several mechanisms could be affected, including defects in mRNA translation, stability of protein, or degradation of the Aire protein. These data strongly indicated that the loss of a single TRA (i.e. OVA peptide) licenses the *de novo* generation of autoreactive CD4^{+} T cells during murine aGVHD (Manuscript, Figure 2A). I could demonstrate that emerging OVA-specific T cells were still functional and OT II^{+} T cells had a non-anergic phenotype (Manuscript, Figure 2C) and remained their capacity to respond to their cognate antigen *in vitro*. The finding that the

peripheral OT-II$^+$ T cells represented effector T cells and not conventional T_{reg}, was important and indicated that they were capable of responding to their antigen. This observation was supported by the finding that T-cell proliferation was strong in response to *in vitro* exposure to OVA peptide and syngeneic APC (Manuscript, Figure 2D).

6.1.1 Functionality of *de novo* generated autoreactive T cells?

It is of great importance to address the question whether these OVA-specific T cells that leave the thymus suffice to cause autoimmunity as a consequence of aGVHD. Since mOVA is expressed under the rat-insulin promoter, the question arose whether OT II$^+$ T cells would infiltrate the pancreas, induce damage of islet cells and cause type 1 diabetes. I investigated if emerged OVA-specific T cells infiltrated pancreatic LN in these recipients but I did not observe a significant infiltration. In this respect, two previous publications argued against an islet-destructive function of CD4 cells alone.[213,205] Kurt *et al*. could demonstrate that even high numbers of OT-II cells alone would not induce islet infiltration and ensuing diabetes in RIP-mOVA mice in the absence of OT-I cells. Moreover, impaired negative selection in Aire-deficient RIP-mOVA x OT-II mice did not suffice to cause diabetes, in contrast to OT-I. Since we focused on OT-II cells in our experimental setting, I hypothesize that emerging OT-II cells alone would not mediate islet infiltration and/or destruction. An alternative approach to test *in vivo* if the emerging cells are still functional could be by the injection of EL4-OVA cells into the RIPmOVA recipients, expecting the escaping OVA-specific T cells to attack the tumor and eliminate it. Unfortunately, I was limited in the experimental set up because the EL-4 OVA cell line was made on a MHC-I background and there was not sufficient time to genetically alter them for use as a potential target for MHC-II restricted CD4$^+$ OVA-specific T cells.

In summary, I provided direct evidence in transgenic mice that *de novo* production of TRA-specific T cells during aGVHD is a consequence of impaired ectopic TRA expression that results from a diminished mTEC^{high} cell pool. Our data therefore indicate that a functional compromise of the mTEC compartment may link alloimmunity to the development of autoimmunity which is a characteristic event of cGVHD.

6.1.2 A mechanistic link for the transition from acute to chronic GVHD

Based on my findings and others, I propose the following mechanistic link between aGVHD and cGVHD: After allo-HSCT BM-derived T-cell progenitors migrate to an intact host thymus where they undergo normal positive selection. Being positively selected, thymocytes migrate to the

medulla, undergoing negative selection, where developing T cells are exposed to self-antigens, including TRA. The ability of Aire^{+}mTEC^{high} to build a reservoir of a large array of TRA is essential for the negative selection of self-reactive T cells. TCR ligation of these TRA with high affinity leads to clonal deletion of the developing thymocyte (see also chapter 2.2.2.3). However, the expression of an individual TRA is restricted to a small subset of Aire^{+}mTEC^{high}. The high level of heterogeneity between mTEC subpopulations hence explains why the size of the Aire^{+}mTEC^{high} compartment contributes to TRA diversity. Ectopic TRA expression is a stochastic process, where different mTEC^{high} cells express different TRA on their surface, as illustrated in Figure 25. Therefore, a comprehensive mosaic of TRA is only achieved by the complete mTEC^{high} pool. This mechanism ensures an efficient deletion of a highly diverse self-reactive TCR repertoire. Normally, mature T cells that have survived negative selection are exported to the periphery as naïve T cells, being self-tolerant but pathogen-specific. During aGVHD alloreactive donor T cells (T_{allo}) infiltrate the thymus and specifically attack Aire^{+}mTEC^{high} cells, however, leading to cell death and consequently a diminished transcription of a smaller array of TRA (Figure 25, blue arrow, blurry Aire^{+}mTEC^{high} cell). Hence, proper negative selection cannot occur anymore, leading to the *de novo* generation of autoreactive T cells which can then be released into the periphery. This proposed mechanism is supported by another study from our laboratory where it was shown that these TRA whose expression levels were reduced as a consequence of aGVHD represented proteins that are specifically expressed in known target tissues of cGVHD, including i.e. salivary glands (e.g. salivary protein), liver (e.g. urinary protein) and lung (e.g. secretoglobin).[134]

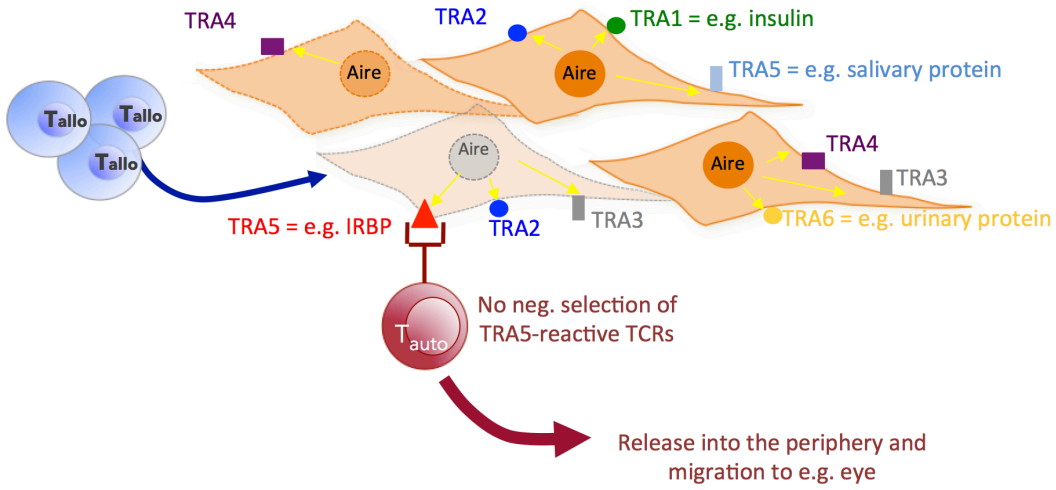


Figure 25: ***Proposed etiological link between aGVHD and cGVHD.*** *During aGVHD, alloreactive T cells infiltrate the thymus and target TRA-expressing Aire^{+}mTEC^{high} cells. This includes the antigen-presentation of i.e. interphotoreceptor retinoid-binding protein (IRBP), a target protein during cGVHD. If there is a lack of IRBP-expressing mTEC, de novo generated autoreactive T cells against IRBP emerge from the thymus and might induce uveitis – a syndrome in cGVHD.*

Although these data highly suggest for a possible mechanistic link between aGVHD and cGVHD, it should be noted that *de novo* generated autoreactive T cells that emerge from the thymus may not be sufficient to cause an autoimmune syndrome typical for cGVHD. This fact may be due to additional control mechanisms that regulate autoimmunity in the periphery. One example is the presence of natural T_{reg} that are generated in the thymus and suppress peripheral autoreactive T cells. Since the thymic microenvironment is damaged during aGVHD, there is much evidence that not only the elimination of autoreactive T cells is affected but also the generation of T_{reg} cells is impaired. If such a mechanism was true, this would argue for a "2-hit model"[139] (see also chapter 2.2.4). Here, development of cGVHD is caused by the emergence of autoreactive T cells from the thymus to the periphery "Hit 1" and the parallel deficiency of T_{reg} "Hit 2".

An alternative working model that helps to clarify how the loss of *Aire* is leading to spontaneous autoimmunity can be the use of Aire deficient mice. Mice that lack *Aire* demonstrate an impaired negative selection process and mirror the proposed mechanism occurring during aGVHD. It was previously reported that Aire deficient mice spontaneously develop an autoimmune response against the posterior chamber of the eye, resulting in uveitis – a severe inflammation in the eye.[205] It was also demonstrated that the target of the immune infiltrates in the eye is a specific Ag, namely the interphotoreceptor retinoid-binding protein (IRBP).[214] Moreover, it was shown that

the IRBP antigen is expressed as TRA by Aire^{+}mTEC^{high} and tightly regulated within the thymus.[214] Although the development of uveitis is mainly induced by auto-Ab against IRBP, several studies could show that athymic nude mice with aire-deficient thymii did develop uveitis, indicating that the eye disease is indeed mainly driven by effector T cells in Aire$^{-/-}$ mice.[114,205] Interestingly, a commonly observed feature of cGVHD patients is ocular GVHD characterized by uveitis that occurs in 40-60% of patients that undergo allo-HSCT.[215] It may be hypothesized that chronic eye infection in cGVHD patients indeed depends on the impaired negative selection of autoreactive T cells against IRBP. In this scenario, lack of IRBP expressing Aire^{+}mTEC^{high} during aGVHD leads to autoreactive T cells that escape negative selection and can be released into the periphery inducing uveitis (see Figure 25). In order to test this hypothesis, I performed a qPCR for IRBP detection in my aGVHD model. Unfortunately, mRNA was undetectable. An easier way to detect autoreactive T cells against IRBP within a T-cell pool with a polyclonal repertoire would be with a tetramer or pentamer reagent.[214] Such experiments were, however, out of the scope of this thesis project due to time limitations. In the future, the identification of the specificities of autoreactive effector T cells in cGVHD may allow to test whether such mechanism operates not only for a surrogate TRA but is universal for thymic ectopic expression of those TRA that are present in tissues known to be targets of cGVHD. I could not answer this particular question during my PhD and there is a need for further investigations.

Other previously reported observations provide additional evidence that antecedent impaired negative selection during aGVHD might act as predisposition of cGVHD.[55,135,137]If this mechanistic link is true, this would in fact explain, why cGVHD is correlated with lower leukemic relapse.[216] Many of the TRA that are expressed by Aire^{+}mTEC^{high} are also present in tumors – thus within the autoreactive T-cell pool that recognizes TRA with too high affinity there are also tumor-recognizing T cells present and get eliminated by negative selection.[217] It was previously reported that manipulation of negative selection by the blockade of RANKL enhanced anti-tumor immunity.[216] These results support the assumption that during aGVHD, thymic negative selection fails. Thus, tumor-specific T cells may survive central tolerance and can be released into the periphery, where they induce cGVHD but at the same time reduce the risk for tumor relapse.[216]

To summarize, my data provide a mechanism for how autoimmunity may develop in the context of aGVHD. This mechanism is based on a contracted mTEC cellularity and consequently impaired TRA representation due to altered gene expression. In conclusion, the weakened platform for negative selection might provide the explanation for the emergence of autoreactive T cells seen in the murine transplantation models. As a consequence, therapy for cGVHD has

traditionally been directed at suppressing the donor anti-recipient immune response. There is currently no effective treatment for acute and chronic GVHD apart from immunosuppressive drugs that unfortunately enhance the risk for infections and inhibit an advantageous anti-tumor immunity. In an effort to find alternative solutions, I focused in the second part of my thesis on an approach that included cytoprotection of the TEC compartment via interfering with T-cell migration patterns from SLO into the thymus using the administration of KRP203.

6.2 $S1PR_1$ agonist KRP203 in the context of allo-HSCT

6.2.1 Relevance of the KRP203 study

New approaches are sought that specifically prevent aGVHD but do not interfere with T-cell immunity directed against cancerous cells. Since both beneficial anti-tumor effects and deleterious anti-host effects responses are based on T-cell immunity, a generalized immunosuppression will inhibit both effects equally. The separation of GVT and GVH responses is hence a major challenge to both transplant immunologists and physicians. Recent work has revealed a novel concept for GVHD prophylaxis.[181,195] This approach uses interference with early T-cell trafficking and is based on the concept that inhibition of donor T-cell migration from SLOs to peripheral target organs reduces GVHD. S1P receptor agonists such as KRP203 or FTY720 have shown beneficial therapeutic effects in preclinical models of GVHD.[182,188]

As introduced in chapter 2.3, a parallel clinical study had been initiated prior to initiation of our preclinical research (ClinicalTrials.gov Identifier: #NCT01830010). This interventional study is sponsored by Novartis Pharmaceuticals and is entitled: "A Two-part, Single- and Two Arm Randomized, Open-label Study to Evaluate the Safety, Tolerability, Pharmacokinetics and Efficacy of KRP203 in Patients Undergoing Stem Cell Transplant for Hematological Malignancies". Primary outcome measures: Number of participants with adverse events as a measure of safety (111 days). Secondary outcome measure: GVHD-free, relapse-free survival (1-2 years posttransplantation). The study is still in the recruiting stage and a primary completion date of January 27, 2020 is targeted. For this reason the data are not yet reportable. By using a two-pronged approach of our KRP203 study that combines preclinical and clinical investigations at the same time, I believe that there will be a reciprocal benefit for both the scientific and the clinical perspectives. The experimental data obtained herein are expected to provide a basis for the rational use of KRP203 in the clinical setting.

6.2.2 KRP203 administration in naïve mice in the absence of aGVHD

An optimal administration protocol for KRP203 for preventing or treating GVHD has thus far not been reported. The conventional readout to analyze lymphocyte migration is the analysis of lymphocyte counts within the PB. Since the $S1PR_1$ agonist Fingolimod is approved as an oral drug, I first compared oral administration of KRP203 versus i.p. injections as the latter is more convenient to apply in mice (data not shown). I did not observe any differences between both application forms, therefore I decided to use i.p. injections for future experiments. However in patients, KRP203 is still suggested to be given in an oral form. The recommended dose was reported to be in a range between 0.3 - 3mg/kg. Therefore I tested several doses and observed the highest reduction in lymphocyte counts with 3mg/kg. It is important to note that I never reached an absolute block in lymphocyte migration but a large reduction in absolute cell numbers and frequencies was always observable (Figure 9). Due to the fact that lymphocyte reduction was only observed at 1 and 2 days after the first injection, I argued that prophylactic administration might be crucial to diminish donor T-cell migration in murine allo-HSCT recipients. Moreover, the fact that lymphocyte numbers returned to normal values again within 2 days after WD, I decided to apply KRP203 every 2nd day. An interesting but not surprising finding was the fact that CD4SP and CD8SP T cells did accumulate in the thymus of naïve mice under KRP203 administration (Figure 10), suggesting for either higher proliferation in thymocyte subsets or, alternatively, a block in thymic egress, as it was already reported for FTY720.[163,164] I could detect proliferation capacity by BrdU administration and observed that proliferation tended to be increased among SP cells. These results indicated that the accumulation of mature T cells indeed might be due to an increased proliferation and not due to an impairment in thymic export when mice received continuous KRP203. However, the difference was not statistically significant (Figure 11). Interestingly, I did not observe an accumulation of thymocytes when I investigated the effect of KRP203 in an allo-HSCT mouse model where mice experienced TBI (Figure 12). Moreover, I found that engraftment of HSC-derived T-cell progenitors to the thymus remained normal with regard to absolute cell numbers and frequencies of ETP (Figure 13). Also, intrathymic T-cell development was not different in mice that received KRP203 compared to control mice within all DN stages. Lastly, I did not see any block in the export of mature CD4SP or CD8SP T cells, as they were also present in SLO 3.5 and 6 weeks post allo-HSCT (Figure 12). An explanation for this contradictory observation between unirradiated and lethally irradiated recipients could be the fact that the thymic importation is a highly regulated periodic event.[210] The thymus does not replenish itself since it does not contain any self-renewing thymic progenitors. Thus, a continuous importation is necessary to sustain a normal T-cell output. When I continuously applied KRP203 in naïve mice, T-cell numbers were likely to be already high, resulting in full intrathymic niches.

Occupied niches within the thymus are believed to negatively regulate thymic import of early progenitors via a feedback mechanism.[210] Indeed, it was shown that P-Selectin expression is an essential factor in this feedback mechanism and the size of the progenitor pool is dependent on P-Selectin expression on endothelial cells.[87] Thus, to address the question if egress is blocked (followed by a negative feedback loop of thymic import) I analyzed P-Selectin expression on endothelial cells in naïve mice. I did not find a significant difference when compared to untreated mice after 1 consecutive week of KRP203 administration although I did see a trend towards reduced P-Selectin expressing endothelial cells (Figure 13). Thus, in the conditioned model, where recipients underwent TBI, it could be that there was still space to fill up thymic niches and the tightly regulated import and export were not affected under KRP203 administration post allo-HSCT. In addition to that, and as already mentioned before, I never saw a complete block of lymphocyte migration in the PB (chapter 6.2.2). This might also be true for thymic input but also export from the thymus.[210] Lastly, independent lines of preclinical evidence suggest that homing and engraftment of donor cells are not hampered in FTY720 pretreated animals receiving allo-HSCT.[218] However, the combination of a CXCR4 antagonist and an $S1P_1$ agonist was shown to have the potential to mobilize HSC's in normal donors,[219] suggesting a direct effect of S1P receptor modulation on stem cells homing which might translate into impaired engraftment in humans.

6.2.3 KRP203 reduces thymic donor mature T-cell infiltration but still provides intrathymic donor BM-derived T-cell development

aGVHD is initiated by alloreactive donor T cells, which target a restricted set of host tissues. In preclinical models, FTY720 has been demonstrated to markedly reduce aGVHD in multiple clinically relevant murine strain combinations.[179,181] The failure of FTY720 to treat established advanced GVHD in both mouse and dog models[220] underlines the importance of starting treatment before symptoms occur. Therefore, the use as a prophylactic drug seems to bear the greatest clinical potential. To investigate the effect of KRP203 on aGVHD prevention, I used of the non-ablative *B6→BDF1* transplantation model. This model allowed me to distinguish pathophysiological influences on the TEC compartment caused by aGVHD from those that resulted from cytoreductive therapy. Thus, unirradiated mice were transplanted with T cells from a haplo-ID donor and developed aGVHD within 2 weeks. In this model, the prophylactic administration of KRP203 caused indeed a decrease in total numbers and frequencies of thymus-infiltrating donor mature T cells (Figure 15). As a consequence of diminished thymic injury, I observed that pro-inflammatory cytokines were also decreased as I measured reduced secretion of TGFβ in the thymus when mice received prophylactic KRP203 (Figure S1). Importantly, I could

confirm by immunohistochemistry as well as flow cytometry that the diminished intrathymic anti-host immune response resulted in the preservation of the $Aire^{+}mTEC^{high}$ pool (Figure 19). Absolute cell numbers of $Aire^{+}mTEC^{high}$ remained normal, as they were comparable to mice without aGVHD. I also investigated the effect of KRP203 on thymic donor T-cell infiltration in two additional murine allo-HSCT models including a lethally irradiated and fully MHC-mismatched model and a lethally irradiated miHA mismatch model. In both models, I could confirm that KRP203 prophylaxis decreased frequencies and absolute cell numbers of donor T cells infiltrating the thymus. These mice had, as a direct effect of lower pro-inflammatory T cells present, a preserved TEC compartment and increased numbers of $Aire^{+}mTEC^{high}$ cells when compared to mice that developed aGVHD but were left untreated. These data directly proved that TRT such as aGVHD but also conditioning regimen cannot harm the thymus to the same extent, when KRP203 is given prophylactically and continuously.

Having seen a beneficial effect of KRP203 on thymic function, I wondered whether KRP203 also influenced thymus-dependent T-cell regeneration in the recipient following allo-HSCT. As already observed in the MHC-mismatched model without aGVHD, I found that prophylactic and continuous KRP203 application did not impair the intrathymic T-cell maturation process even in the presence of aGVHD. An overview of the effect of KRP203 on several host compartments in all different mouse models tested is summarized in table 2 (see appendix). I did not test whether the peripheral T-cell pool was affected by KRP203 in the presence of aGVHD later than 2 weeks (Figure 18). However, data from lethally irradiated mice that received TCDBM (no GVHD) from MHC-mismatched donors showed normal peripheral T-cell development 3.5 and 6 weeks post allo-HSCT (Figure 14). I therefore concluded from these results that KRP203 did not have a detrimental effect on thymic export of new naïve T cells. Whether this resulted following allo-HSCT in a normal peripheral T-cell pool remains to be investigated.

6.2.4 KRP203 increases T_{reg} numbers

There is much evidence that T_{reg} have a beneficial function in GVHD prevention.[221] Based on the observation that CD4SP T cells are increased in the thymus during KRP203 administration, I asked whether the T_{reg} subset also had higher cell numbers. For that reason I investigated the frequencies of $CD4^{+}CD25^{+}FoxP3^{+}$ T_{reg} in the same experimental setting as described above (chapter 6.4). T_{reg} were indeed increased in frequencies among $CD4^{+}$ T cells when mice received prophylactic KRP203 administration (Figure S2A). Moreover, I analyzed frequencies of host T_{reg} in the periphery since $S1PR_1$ agonism is expected to trap T cells in SLO. Mice that had clinical signs of aGVHD had -as expected- decreased frequencies of T_{reg} whereas prophylactic KRP203 administration increased T_{reg} frequencies in spleen and LN in comparison to mice with aGVHD

but without treatment (Figure S2B). This finding was, however, not surprising since it was already reported that FTY720 promotes $FoxP3^{+}T_{reg}$ cell differentiation from thymic T_{reg} precursors. This observation was explained by the fact that functional antagonism of $S1PR_1$ activates mTORC1 signaling which triggers Smad3 activation and hence antagonizes T_{reg} differentiation.[183] Thus, by the use of $S1PR_1$ agonism, this effect should be reversed.

In total, the experiments demonstrated that prophylactic application could significantly reduce infiltration of donor T cells to the thymus and subsequently diminish the secretion of pro-inflammatory cytokines. This in turn had an advantageous effect on the thymic microenvironment and provided normal donor-BM derived T-cell development as a normal TEC compartment was preserved. Concomitantly, host T_{reg} were increased in frequencies suggesting for an additive role in the prevention of aGVHD which remains to be investigated.

6.2.5 Withdrawal of KRP203 reduced T-cell migration only for a limited time frame

As an observation that is likely clinically relevant, the WD of KRP203 administration resulted in diminished import of donor-derived T cells to the thymus at 1 and 2 weeks after WD (Figure 17). In parallel, the size of the mTEC compartment remained normal with regard to absolute cell numbers. In addition to this observation, I detected (in collaboration with Phil Smith, Novartis) that mice that developed cGVHD had much higher probability to survive when KRP203 was given either continuously (from day-1 until day 60) but also when KRP203 was given prophylactically for 1 week and then withdrawn.

These results suggested that, although low numbers of alloreactive donor T cells were present in the thymus over 2 or 3 weeks during continuous KRP203 administration, they were not sufficient in numbers to induce damage in the TEC compartment. However, it remains to be elucidated why donor T-cell numbers in the thymus remained low 1-2 weeks after WD, although I observed in previous experiments that T cells resumed migration 2 days after WD as they were detectable in the PB. One explanation could be the fact that KRP203 might rather be a $S1PR_1$ modulator than agonist.[222] Under physiological conditions, S1P (acting as an $S1PR_1$ agonist) induces only the internalization of its receptor $S1PR_1$, which then recycles back to the cell surface within hours.[223] The phosphorylated form of Fingolimod (FTY720) however works as a $S1PR_1$ functional antagonist, where the internalized receptor is degraded by the proteasome.[185,222] This results in a long-term absence of the receptor and thus a long-term effect until there is *de novo* production of the receptor, which needs days, rather than hours.[223] My results hence strongly suggest degradation of the receptor after long-term internalization and not recycling and resensitization. Thus, after KRP203 administration for 2 weeks, the effect might endure for another 1-2 weeks

after WD. This is a very relevant observation for the translation into the clinics. These results are important indicators for duration of KRP203 administration and suggest for a prophylactic and continuous period of administration during the first weeks after allo-HSCT, until there is full regeneration of a new T-cell compartment.

6.2.6 Prophylactic but not therapeutic administration prevents thymic T-cell infiltration during aGVHD

I did not observe a beneficial effect of KRP203 on aGVHD prevention, when KRP203 was given therapeutically instead of prophylactically (Figure 16). When I started drug application at day 7 after allo-HSCT, I detected high numbers of donor mature T cells present in the blood and I observed elevated numbers of pro-inflammatory donor T cells in the thymus in mice with aGVHD (Figure 16). This observation could indicate that alloreactive donor T cells invade the thymus within the first 7 days post-HSCT. As a consequence, the therapeutic administration effectuates a reduced ability for T cells to egress from SLO, leading to an accumulation of donor mature T cells that induced even more harm. This observation was reflected by the fact that the $Aire^{+}$mTEC compartment was not preserved during aGVHD when KRP203 was applied therapeutically (Figure 17). Furthermore, I observed that therapeutic application also resulted in very low frequencies of host T_{reg}, thus intensifying symptoms of aGVHD (Figure S2B).

Taken together, I concluded from my experiments that prophylactic and continuous administration of KRP203 maintained a normal thymic stromal microenvironment and hence normal thymopoiesis to occur following allo-HSCT.
These results are pertinent for several reasons: The use of $S1PR_1$ agonist allowed me to determine the threshold for donor T-cell numbers to infiltrate the thymus without inducing irreversible damage to the thymic environment, including the TEC compartment. Moreover, I could define a time frame for how long alloreactive T cells can stay in the thymus without worsening thymic damage. Lastly, I found an optimal dose that reduces the quantity of alloreactive donor T cells to an amount that thymic damage is not induced while on the same time, BM-derived T-cell precursor still can engraft in the thymus and egress to the periphery.

6.2.7 Negative selection of autoreactive T cells is preserved upon KRP203 administration

My observation that the TEC compartment was preserved with regard to absolute cell numbers during aGVHD by prophylactic KRP203 administration led me to the next question whether the preserved mTEC compartment also remained functional in its ability to eliminate strongly self-

reactive T-cell clones. Thymocytes that are highly self-reactive induce Helios and PD-1[101,102] as response to self-antigens that are particularly expressed by the $Aire^{+}mTEC^{high}$ compartment. The surface expression of Helios and PD-1 thus helps mTEC to recognize highly self-reactive T cells and to eliminate them.[101] The expression of Helios by $CCR7^{-}TCR\beta^{+}$ thymocytes therefore provides a direct way to enumerate T cells that carry a strong self-reactive TCR and are susceptible to deletion. I detected normal numbers of autoreactive T cells that will be negatively selected by the induction of Helios in mice without GVHD and mice that received KRP203. Interestingly, mice that did develop aGVHD had, against my expectation, only low frequencies of Helios expressing cells, as I expected higher frequencies of autoreactive T cells to be present (Figure 20). A reason for this observation could be that during aGVHD, autoreactive T cells indeed can escape negative selection and not even induce Helios or PD-1 as response to antigen-presentation because of lacking TRA-expressing $Aire^{+}mTEC^{high}$. These data presented a new finding that indeed negative selection is directly affected during aGVHD via the lack of Helios and PD-1 induction by autoreactive T cells. Moreover, I found that this mechanism could be preserved by the administration of KRP203. In the presence of aGVHD, *de novo* generated autoreactive T cells can be released to the periphery.[137,138] Based on the observation that central tolerance is still provided by KRP203 administration during aGVHD, I wondered whether this directly led to a diminished release of *de novo* generated autoreactive T cells. Indeed my data indicated that KRP203 administration diminished, through the preservation of $Aire^{+}mTEC$ and subsequent capacity to express OVA in the thymus, the emergence of *de novo* generated OVA-specific autoreactive T-cell clones to the periphery (Figure 21).

Taken together, my data suggested that the lack of abundant numbers of host-reactive T cells rendered the transition from acute, inflammatory disease to autoimmune-like syndromes such as cGVHD more unlikely. My results confirmed that prophylactic KRP203 administration was able to prevent loss of $Aire^{+}mTEC^{high}$ cells. These cells serve as a platform for central T-cell tolerance induction. My data raised the possibility that preventing mTEC damage had averted the export of autoreactive T cells that are believed to be responsible for the transition from aGVHD to the autoimmune form of cGVHD.

6.2.8 KRP203 – a drug for cGVHD?

There is evidence that the advantageous effect of KRP203 on the thymic environment during aGVHD could also have a function in cGVHD prevention. Wu *et al.* recently established a cGVHD mouse model where mice develop symptoms of cGVHD within 60 days. Here, antecedent thymic damage leads to an impaired negative selection process due to mTEC damage

and the generation of autoreactive T cells.[137] To test if KRP203 prevents symptoms of cGVHD, I chose to establish that model in our lab (Figure S3A). However, I did not observe thymic damage to the same extent as published (Figure S3C). Furthermore, I took serum from these mice and tested for auto-Ab production against salivary glands and skin on tissue from $RAG^{-/-}$ mice. I did detect low amounts of anti-dsDNA auto-Ab production (~10%) in the salivary glands in mice that developed cGVHD and less auto-Ab in mice that received TCDBM only (Figure S3D). However, these were only preliminary results and hence this issue needs further investigation. Another approach to test if auto-Ab are produced, may be by the isolation of the retina of $RAG^{-/-}$ mice and test if the serum of mice with cGVHD contains auto-Ab against IRBP. Moreover, one could test if autoreactive T cells are present against IRBP. Future experiments may wish to address this question which is beyond the scope of my PhD.

There are patients who develop cGVHD symptoms without antecedent aGVHD symptoms.[40] It still could be possible that thymic damage could already occur before symptoms of cGVHD arise. In these patients, KRP203 administration could also be helpful since it could block (if it is applied at higher doses) or at least reduce egress of autoreactive T cells that already might have developed in the thymus. Hence, KRP203 could temper autoimmune symptoms present during cGVHD. However, this hypothesis needs to be tested. A possible mechanism for KRP203 administration in cGVHD is illustrated in Figure 26.

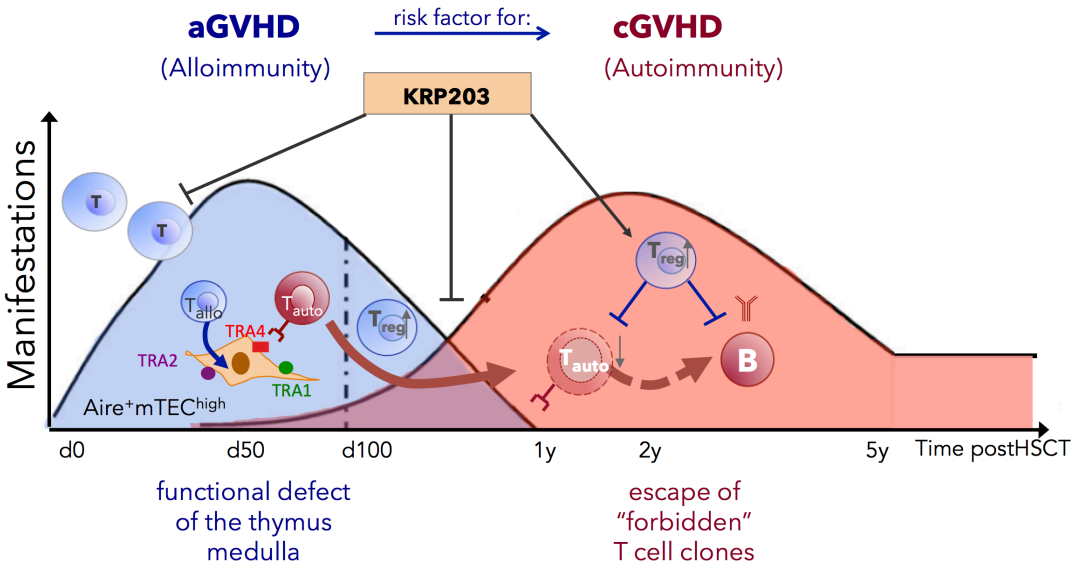


Figure 26: ***Possible mechanism for KRP203 application in cGVHD.*** *KRP203 blocks donor T-cell infiltration into the thymus and thus preserves the $Aire^{+}mTEC^{high}$ compartment. This may allow a proper elimination of autoreactive T-cell clones and subsequently no emergence of autoreactive T cells. In addition, T_{reg} numbers in thymus and periphery are*

increased upon KRP203 administration. When KRP203 would be applied in higher doses, thymic egress of already generated autoreactive T cells might be blocked.

6.2.9 Out of the thymus – what happens in the periphery?

KRP203 still provides an anti-tumor response

Although previous studies have demonstrated that GVT is mostly retained under FTY720 administration, mechanistic information as to where and how anti-tumor immunity is functional was lacking. I hypothesized that lymphomas were better targets of donor T cells under the KRP203 umbrella because this tumor resides in SLO. In contrast, as control of disseminated leukemia requires trafficking of donor T cells, an anti-tumor immunity to those tumors would be possible only after drug WD. Indeed, I observed that the tumor could be cleared with KRP203 administration (Figure 24). In the same model but without tumor development, I could detect donor mature T cells being present within SLO during KRP203 administration (Figure 22). These results indicated that an optimal dosage and application protocol of KRP203 could effectively retain a sufficient number of T cells in SLO when desired. However, I did not test whether this was also true for other disseminating tumors. However, WD of KRP203 might allow for anti-leukemic effects upon release from SLO. These data were not generated during my thesis but may be generated by following up incidences of relapse in patients that participate the parallel clinical trial.

Previously published findings showed that naïve T cells but not effector memory $CD4^+$ (T_{em}) and $CD8^+$ (T_{em}) cells nor central memory T cells (T_{cm}) induce GVHD while $CD4^+$ T_{em} cells mediated GVT effect against leukemia.[224] Moreover, the $CD8^+CD44^{high}$ memory T cell subset containing central memory and effector memory cells was able to exploit the GVT effect against lymphoma without inducing GVHD.[24,225] In parallel, Mehling *et al.,* showed that naïve T cells and T_{cm}, which home trough LN, were significantly reduced upon FTY720 administration, whereas T_{em}, that preferentially screen tissue, were not as affected as naïve T cells and T_{cm} cells.[226] These data support my observations that tumor eradication is still provided under KRP203 administration. Activated T cells with cytotoxic activity are able to kill the tumor and are not "trapped" to the same amount as naïve T cells under KRP203 administration – an observation that may even be better for GVHD prevention.

Another potential mechanism for anti-tumor immunity might be the tumor killing by NK cells. NK cells are known to provide strong anti-tumor immunity[227] and were reported to be only partially affected by $S1PR_1$ agonists.[170,178] I also checked for different lymphocyte subsets that were affected by KRP203 in naïve mice and did not observe a block in NK cell migration. Thus, I

argued that the same was true in my tumor model and NK cells supported the T cells in the elimination of the tumor.

Another important question was raised whether the lymphocytes that are trapped within the SLO, still remained functional. To test whether residual T cells within SLO had an effector phenotype and did not become anergic upon continuous KRP203 application, I first measured folate receptor 4 (FR4) and CD73[228] expression on peripheral T cells in LN of mice that developed aGVHD with or without KRP203 and in mice without GVHD. In the control group, the majority of $CD4^+$ T cells expressed FR4 and CD73 at very low levels indicating a non-anergic phenotype (Figure S4A). During aGVHD, a higher frequency of T cells expressed FR4 and CD73 at high levels, a typical feature of anergic cells (see Appendix, Manuscript Figure 2C). Continuous KRP203 administration resulted in only small numbers of $CD4^+FR4^{high}CD73^{high}$ cells (Figure S4A). In addition to the phenotypical characterization of anergic cells, I also tested their function via analyzing the capacity of IFNγ secretion. I observed that during aGVHD phenotypical anergic cells were still able to secrete IFNγ independent of KRP203 administration (Figure S4B). These data showed that only low numbers of T cells became anergic with respect to surface marker expression and cytokine secretion, when KRP203 was continuously applied for 2 weeks. I did not answer the question whether these cells remained functional with regard to their ability to fight pathogens. This question is of great importance as opportunistic infections are a frequent setback for allo-HSCT patients. Microbial infections are mostly controlled by the migration of effector T cells from LN to the site of infection. Since it was shown that T_{em} are not as affected as naïve T cells by $S1PR_1$ agonists,[226] it is likely that the ability to clear infections is provided despite KRP203 administration. Moreover, innate cells that are not trapped by KRP203, might still serve as first-line protection.

Lastly, I asked whether $S1PR_1$ agonism led to lymphopenia, as S1P as well as the phosphorylated form of FTY720 were reported to subsequently internalize the receptor.[184]
[185,186] However, I could exclude the latter mechanism since in a transplantation setting, lymphocyte retention in LN does not induce subsequent lymphopenia as it was shown after allo-HSCT that the peripheral T-cell expansion is not sufficient to replenish the T-cell pool which needs thymic output to be complete.[78]

6.3 Translation of preclinical findings to the clinical application – general considerations

Although there was an overall increased survival probability after allo-HSCT over the years, there is still room for improvement.[229] While many specific mechanistic insights have been gained by experimental work, not a single proposed mechanistic pathway that has been identified experimentally has successfully translated into clinical reality. The PhD project was situated at the interphase of basic transplantation immunology and clinical stem cell transplantation. Using a synthetic drug of a known class of successful experimental GVHD prophylaxis that is also being used in a clinical trial that we have initiated in June 2013, I have aimed to close gaps in the field and to facilitate the clinical implementation of S1PR agonism as a prophylactic principle, thereby preventing the suffering of many patients.

In 2014, Markey *et al.* suggested considerations that should be taken in order to evaluate the translation of new therapeutic interventions into clinical practice (table 1).[230]

Will a new therapeutic strategy translate into the clinic?
1. Is the pathway in question still active in patients (or animals) receiving immune suppression? If not, does preclinical evidence suggest the inhibition of the pathway in isolation will be feasible and more efficacious than current immune suppression?
2. Is the effect large (complete) rather than incremental (partial)?
3. Does the intervention inhibit GVHD at multiple stages of its pathophysiology, and preferably early?
4. Is the pathway active in both class I– and class II–dependent GVHD? If not, it should at least not have opposing effects in CD8 and CD4 T-cell–dependent GVHD.
5. Are there potential toxicities in humans that might reasonably not be evident in the animal model?
6. Are there detrimental effects of the intervention on GVL and pathogenic-specific immunity and if so is this manageable and outweighed by the potential benefits in treating GVHD?
7. Bearing in mind that pharmaceutical companies have minimal interest in BMT as a sole market, is the pathway operative in other disease settings such that the generation and development of reagents to the clinic is likely to be commercially viable?

Table 1: ***Predicting the translation of preclinical therapeutic strategies.*** *According to Markley et al. this list of questions helps to predict if preclinical therapeutic strategies will be translated into the clinics.*

Although the compound KRP203 is already being tested in a parallel clinical trial, I evaluated the potential for this compound to be used for GVHD prevention for higher clinical trial phase. Based on the questions that were listed in the table, KRP203 seems to be a promising new therapeutic strategy for preventing aGVHD. First of all, KRP203 restricts T-cell migration by interfering with

the S1P-$S1PR_1$ axis, a pathway that still might be active in patients that receive immunosuppressive drugs, which only dampen T-cell activation but not migration (REF). The effect, if applied prophylactically, is large and systemic. Interference by KRP203 inhibits (thymic) aGVHD at a very early stage of its pathophysiology and might even have advantageous long-term effects as it may mitigate cGVHD symptoms by reducing the emergence of autoreactive T cells. $S1PR_1$ is expressed by both $CD4^+$ and $CD8^+$ T cells and thus interferes with MHCII and MHCI downstream signaling pathways, respectively. Possible toxicities that can arise in patients - but were not evident in animals - will be revealed in the parallel clinical trial. Since similar S1PR agonists are Food and Drug Administration (FDA)-approved and widely used for other disease, it is likely that KRP203 has no detrimental side effects in humans. As I observed for lymphoma, the GVT effect remained active under the KRP203 umbrella. However, I could only test this issue for a lymphoma tumor while it remains unclear how the tumoricidal activity will be for disseminating tumors. Moreover, to this time point, I could not answer the question, if pathogenic-specific immunity is still provided. Nevertheless, I hypothesize that the beneficial effects of KRP203 outweigh possible risks for infections or other side effects. Relating to the last point of the table, I think that this compound has high potential to become commercially viable. This contention is supported by the facts that FTY720, a similar drug like KRP203, is already on the market and frequently used for prevention of other diseases (i.e. multiple sclerosis) and moreover, many preclinical trials for different diseases are ongoing with KRP203.

In summary, KRP203 is administered prophylactically as treatment of already established aGVHD is likely too late for intervention with S1P modulation. Co-administration of KRP203 with standard of care treatment used in the peri-stem cell transplant phase (using drugs such as cyclophosphamide, busulphan, methotrexate, fludarabin, cyclosporine or myophenolate), based on pharmacokinetic characteristics of KRP203 and of those drugs, is expected to result in no major interaction. Overall, based on the preclinical and clinical data generated to date, the prophylactic use of KRP203 may be a safe and effective novel modality to successfully prevent severe aGVHD by inhibiting lymphocyte trafficking into the target organs. Before embarking on a large study addressing efficacy, a small trial is proposed mainly to explore initial safety of S1P modulation in this unique patient population. This Phase Ib study is planned as the first step to enable development of KRP203 for the prevention of aGVHD where the unmet medical need is high.

6.3.1 Of mice and men - Mouse models to study human GVHD

In cases when compounds are transferred to clinical trials, there is still the problem that patients differ in variables such as age, underlying disease, conditioning regimens, prophylactic drugs and organ involvement. This heterogeneity of the patient groups makes the evaluation of a trial very complex.[231] The necessity for parallel pre-clinical trials is evident. Experimental animal studies have made major contributions to the understanding of the pathophysiology of GVHD. Thus, preclinical research in experimental models facilitates the translation into the next phase of a clinical trail.[232] The major advantages of using mouse models to study GVHD are the ability to control multiple variables and dissect every step of the disease progress in an accurate and reproducible way.[233] Markey et al. described in 2015 several benefits using murine systems to study GVHD pathogenesis: I) Environmental conditions can be controlled – such as the microbiota or conditioning regimens. II) The transplantation setting can be controlled by using MHC mismatched or miHC mismatched donor/recipient pairs. III) Genetically mutated key molecules can be studied in donor and recipients and within different cell sub-populations. IV) The GVH reaction can be observed in real time and at various time points. V) Therapeutic applications can be tested in clinical relevant mouse models.[230] However, there are also some limitations using animal models for preclinical studies, which are important to note and need to be addressed in the future: One limitation is the bias of several mouse strains to reflect either an isolated CD4 or CD8-dependent response that facilitates the understanding of a MHC class I vs. II response. However, the GVT response requires similar CD4 and CD8-dependent pathways. The bias towards a T_H1 or T_H2 response can also influence the outcome of the disease and varies within different strains such as B6 or Balb/c.[234,235] Another important aspect to improve is to integrate various treatments in one mouse model. It should also be taken into account that most preclinical mouse studies include TBI as the only conditioning regimen. In the future, all of these different factors have the potential to improve predictive values in preclinical experimental mouse models.[232,233,236] These factors highlight why basic research in animal models is further needed in order to better understand the mechanism underlying any disease and the drug in the context of a controlled environment, where all variable factors can be determined.

7. Conclusion & Significance

Allo-HSCT offers an effective treatment for a broad spectrum of malignant and non-malignant disorders. The success of allo-HSCT is not only dependent on the swift regeneration of immune competence and the lasting control of the underlying disease, but also the minimization of transplant-related complications such as aGVHD and cGVHD, respectively which, however, may still develop to some degree in up to 80% of allo-HSCT recipients. Unfortunately, aGVHD and the immunosuppression needed to control disease slow down regular posttransplantation immune regeneration and consequently also hamper efficient anti-tumor immunity. Moreover, aGVHD also presents a risk factor for the development of cGVHD which owns severe autoimmune-like features. Both aGVHD and cGVHD hence can seriously affect a patients' quality of life. Indeed, no therapy or combination of therapies exist at present to effectively prevent clinical aGVHD while at the same time preserving desired immune responses against foreign pathogens and the underlying malignancy.

In the present work, I aimed at the better understanding in animal allo-HSCT models of the mechanisms how autoimmunity may develop as a consequence of aGVHD and how to prevent such pathomechanism. With regard to mechanistic explanations, I extended research initiated in our laboratory that had focused on the role of thymic injury and the resultant loss of central tolerance induction as a cause for *de novo* production of autoreactive T cells during aGVHD. With regard to intervention into this unwanted path, I extended recent work done by others which had revealed a novel concept for GVHD prophylaxis. This approach uses agonists for $S1PR_1$ and is based on the concept that interference with alloreactive T-cell migration from activation sites in SLO to effector sites in peripheral target organs reduces or even prevents aGVHD.

I here demonstrate I) in published data (see Annex) that loss of central tolerance induction via injury to thymic $Aire^{+}mTEC^{high}$ is essential for failure to centrally delete self-reactive T cells and the emergence of *de novo* generated autoreactive T cells in the periphery. II) In unpublished work, I here describe that prophylactic but not therapeutic blockade of donor T-cell trafficking using the specific $S1PR_1$ agonist KRP203 (obtained from Novartis Inc. Basel) reduces donor T-cell migration to the host thymus, thus significantly attenuating thymic aGVHD and the *de novo* production of autoreactive T cells. Moreover, my data confirms that $S1PR_1$ receptor agonism via KRP203 traps alloreactive T cells in SLO and hence maintains the capacity to reject hematopoietic tumors residing in these locations.

The significance of my PhD thesis research is three-fold: I) Basic research: New approaches are required that specifically prevent GVHD but do not interfere with T-cell immunity directed

against tumoricidal cells. The data shown in the present thesis contributes to basic understanding of the cellular and molecular mechanisms responsible for complications of allo-HSCT. The use of experimental mouse allo-HSCT models that are amenable for manipulations was able to answer the question how $S1PR_1$-mediated receptor agonism interferes with other important events that govern in combination the transplantation outcome: thymus-dependent T-cell regeneration and anti-tumor immunity (which cannot be addressed directly in the phase I clinical study in Basel). II) Clinical relevance: The morbidity and mortality of the allo-HSCT procedure remains unacceptably high even 40 years after its introduction. GVHD is the main culprit of the unsatisfying outcome of stem cell transplantation. The PhD project was situated at the interphase of basic transplantation immunology and clinical stem cell transplantation. I have aimed to close gaps in the understanding of the mode of action of the synthetic drug KRP203 and to facilitate its implementation as a prophylactic principle in clinical practice, thereby reducing the suffering of transplant recipients. III) Socioeconomic significance: Today our aim is not only to cure a patient's underlying disease but also to minimize the incidence and severity of transplant-related complications and thus to optimize the patient's quality of life. As HSCT is a high-cost medicine, a steady increase in utilization has put a burden on health care providers. Thus, the successful development of aGVHD prophylaxis approach that spares patients from developing immunodeficiency would be important for economics of transplantation medicine. As economic strength is the main determinant for transplant rate, lower costs would also support a wider use of HSCT for treatment of hematologic malignancies.

8. Reagents

8.1 General buffers and solutions

ACK buffer:	8.29g NH_4Cl (0.15M) 1g $NaHCO_3$ (10mM) EDTA (0.5M) add 800ml bidest water adjust pH to 7.2-7.4 add 1L bidest water
Blocking solution:	PBS 100x Triton X 1% BSA (Sigma) 1% (goat) serum
FACS buffer:	1x PBS 2% FCS
PBS:	8g/L NaCl 0.2g/L KCl 1.78g/L Na_2HPO x $2H_20$ 0.27g/L KH_2PO_4

8.2 Reagents and chemicals

Anti-biotin micro beads: MACS beads	Miltenyi Biotec
Anti-NK cell monoclonal Ab (clone PK136)	BioXCell
Counting Beads	Invitrogen
Cryo Embedding medium	Medite
DNAse I	Roche
Geneticin	Cellgro
Heparin	Braun
Hydromount	National diagnostics
Hygromycin	Invitrogen
Inactivated fetal calf serum (FCS)	Gibco (Lot: 10270)
KRP203	Novartis

Liberase	Roche
Low Tox-M rabbit complement	Cedarlane
D-Luciferin	Promega
Power SYBR Green PCR Master Mix	Life technologies
Ultra Comp eBeads	eBioscience

8.3 Cytokines

Brefeldin A (BFA)	10ug/ml
Ionomycin	500ng/ml
Phorbol myristate acetate (PMA): $C_{36}H_{56}O_8$	50ng/ml

8.4 Cell culture media and supplements

Hybridoma cell lines and ex vivo cell culture:

SF-IMDM powder (Gibco)	3.024g Bicarbonat
	2% FCS
	0.1% kanamycin
	5mg/ml insulin (Sigma)
	0.3% primatone (Sigma
	1% NEAA (Sigma)
	0.1M b-mercapotethanol

A20 luciferase$^+$ cell line (gift from Gang Zhou, Levitsky laboratory, John Hopkins University, US)

Complete RPMI (Sigma)	5% FCS
	400 g/ml geneticin
	200g/ml hygromycin

EL4 OVA cell line:

RPMI 1640 2mM L-glutamine (Sigma)	1.5g/L Sodium Bicabonate
	4.5g/L glucose
	10mM HEPES
	Sodium pyruvate
	0.05mM beta mercapotethanol
	0.4 mg/ml G418 (Geneticin)
	10% FCS

Freezing medium

Cell culture medium	10% DMSO
	20% FCS

8.5 Kits

BrdU Flow Kit	BD Pharmingen (#559619)
$CD8^+$ T-cell enrichment Kit	Miltenyi Biotec
Dynabeads untouched mouse T cell Kit	Invitrogen
FoxP3 Staining Buffer Set	eBioscience

8.6 Cell lines

Cell lines	**Description**
A20 luc^+	B-cell lymphoma cell line expressing luciferase (on Balb/c ($H\text{-}2^d$) background)
EL-4 OVA	T-cell lymphoma cell line expressing the OVA peptide (C57Bl/6 background and MHC I specific)
T24	Hybridoma cell line for α-pan Thy
31M	Hybridoma cell line for α-CD8
RL-172	Hybridoma cell line for α-CD4
HO-134	Hybridoma cell line for α-Thy1.2

8.7 Animal strains

Strain	Definition	Source
129Sv/S1	Wild-type (H-2^b/CD45.2^+/CD90.1^+) with minor-HA mismatch to CD57Bl/6	Jackson
Balb/c	Wild-type (H-2^d/CD45.2^+/CD90.2^+)	Animal Facility DBM Basel
Balb/cThy1.1	Wild-type (H-2^b/CD45.2^+/CD90.1^+)	Animal Facility DBM Basel
BDF1	Wild-type (F1 generation of DBA & B6) (H-2^{bd}/CD45.2^+/CD90.2^+)	Janvier
C57Bl/6	Wild-type (H-2^b/CD45.2^+/CD90.2^+)	Animal Facility DBM Basel
OT II CD45.1 $RAG^{-/-}$	Mice that have a transgenic TCR specific for the OVA peptide expressed on $MHCII^+$ cells (H-2^b/CD45.1^+/CD90.2^+)	Animal Facility DBM Basel
$RAG^{-/-}$	Mice deficient for the recombinating activating gene (RAG)	Animal Facility DBM Basel
RIPmOVA	Transgenic mice that express membrane-bound OVA peptide under the rat insulin promoter (H-2^b/CD45.2^+/CD90.2^+)	Animal Facility DBM Basel

8.8 Antibodies:

Antibody	Clone	Company
CD3	17A2	BioLegend
CD4	GK1.5	BioLegend
CD5	53-7.3	BioLegend
CD8a	53-6.7	BioLegend
CD11b	M1/70	BioLegend
CD19	6D5	BioLegend
CD24	M1/69	BioLegend
CD25	PC61	BioLegend
CD31	390	BioLegend
CD44	IM7	BioLegend
CD45	30-F11	BioLegend
CD45.1	A20	BioLegend
CD45.2	104	BioLegend
CD45R (B220)	RA-3-6B2	Bio Legend
CD62L	MEL-14	BioLegend

CD62P (P-selectin)	RMP-1	BioLegend
CD69	H1.2F3	BioLegend
CD73	TY/11.8	eBioscience
CD90.1	OX-7	BioLegend
CD90.2	30-H12	BioLegend
CD117 (c-Kit)	ACK2	BioLegend
CD279 (PD-1)	29F.1A12	BioLegend
CD326 (EpCAM)	G8.8	BioLegend
Aire	5H12	eBioscience
BrdU	Mebou-1	BD
CCR7	4B12	BioLegend
FoxP3	150D	BioLegend
H2b	AF6-88.5	BioLegend
H2d	SF1-1.1	BioLegend
Helios	22F6	BioLegend
I-Ab (MHC II)	M5/114.15.2	BioLegend
IFNγ	XMG1.2	eBioscience
IL-2	JES6-5H4	eBioscience
Ly51	6C3	BioLegend
Ly6G/Ly6C (Gr-1)	RB6-8C5	BioLegend
NK1.1	PK136	BioLegend
OVA (SIINFEKL)	25-D1.16	BioLegend
Sca-1	D7	BioLegend
TCRβ	H57-597	BioLegend
Ter119	Ter119	BioLegend
TNFα	MP6-XT22	eBioscience
UEA-1	FL-1061	Vector Laboratories
Vα2	B20.1	BioLegend
Vβ5.1/5.2	MR9-4	BioLegend

8.9 Software

FlowJo	Tree Star In., USA
Indigo software	Berthold Technologies
Prism	GraphPad Software, Inc., USA

9. Acknowledgment

First of all, a big thanks to Werner Krenger for your great supervision and patience over the last years. You always supported me and encouraged me with your passion about science. You really taught me a lot! For me, you were not only a boss but also a mentor, which is quite rare nowadays. I learned how to design and perform experiments, but moreover you taught me how to make good (and aligned) presentations, how to present that everyone understands it and how to write scientific articles which not only helped me during my PhD but will also be helpful in the future.

Another special thanks goes to Lukas Jeker and his group from the Molecular Immunregulation lab. Lukas, I'm very thankful that you let me work in your lab. I always appreciated your feedback during the lab meetings and collaborative projects. Most of all, I'm very happy that I could share the lab with your group members who always treated me like we all belong together and I could always rely on. I really appreciated the help of Marianne and Mara, who always gave advise about my project, FACS plots, presentations, but also giving me good recipes for cooking and baking outside the lab. We had a lot of fun and I really enjoy our friendship and Sushi nights. Marianne, thank you for always solving my IT and "starving" problems. Your chocolate and Yogi-tea reservoir is precious. I'm very happy that we could go through most of our PhD-time together. I would say, "A problem shared is a problem halved". I also would like to thank Romina, who also always supported me and is really the first person running, if there is something wrong. We had a lot to laugh and I even enjoyed our lab-cleanings. Also thank you to Caro, Olli, Regan and Helène, for your work and support.

Moreover I am very grateful for Anne, my lab neighbor. I really appreciate our friendship. You are a really strong woman and I'm impressed how you deal with your work, family and friends but always being in a good mood (and that without coffee!). You always motivated me and when I see that you can handle everything at once, I think I will manage too. I also would like to thank you, Mathias. You were suddenly there when I started my PhD and we became such good friends that we spent all the lunch, coffee and beer breaks together. I'm still very thankful for all your help, support and explanations about lab and life.

In addition, I would like to thank my committee members Stephan Krähenbühl, Christoph Hess and Daniela Finke for your scientific input and help during my PhD. Moreover, I would like to thank also all the other friends (Nadège) and colleagues who either helped me with the mice

(Nicole from the Animal Facility) or the FACS (Flow Cytometry Facility) or just gave me good moments to laugh (for example at the PhD Club, Apéros or Cargo Bar).

Last, I would like to thank my family and friends. A big thank you of course goes to my mother and my brother who always supported me on my way – independent in which direction it went. You were always proud of me which encouraged me a lot. I'm thankful that I can always go to you with any problem. Another special thanks goes to Manu (and Nole), who also always supported me and who was tolerating my moods when I was stressed but also sharing excitement about my work. Thank you for you love, making me happy and that you always bring me to smile and encourage me on my way. Lastly, I would like to thank my girls!! You were always there – long time before I started and I know that you will always be there in the future. You were always interested in my work but still, with you, I could also forget about work. You just accept me how I am and you always understood, when I had to leave earlier from vacation or joined later or when I just came for few days because "I need to go to my mice".

10. References

1. Gratwohl A, Baldomero H, Passweg J. Hematopoietic stem cell transplantation activity in Europe. *Curr Opin Hematol.* 2013;20(6):485-493.
2. Passweg JR, Baldomero H, Bader P, et al. Hematopoietic stem cell transplantation in Europe 2014: more than 40 000 transplants annually. *Bone Marrow Transplant.* 2016;51(6):786-792.
3. Rocha V, Wagner JE, Jr., Sobocinski KA, et al. Graft-versus-host disease in children who have received a cord-blood or bone marrow transplant from an HLA-identical sibling. Eurocord and International Bone Marrow Transplant Registry Working Committee on Alternative Donor and Stem Cell Sources. *N Engl J Med.* 2000;342(25):1846-1854.
4. Passweg JR, Halter J, Bucher C, et al. Hematopoietic stem cell transplantation: a review and recommendations for follow-up care for the general practitioner. *Swiss Med Wkly.* 2012;142:w13696.
5. Deeg HJ, Sandmaier BM. Who is fit for allogeneic transplantation? *Blood.* 2010;116(23):4762-4770.
6. Barker JN, Davies SM, DeFor T, Ramsay NK, Weisdorf DJ, Wagner JE. Survival after transplantation of unrelated donor umbilical cord blood is comparable to that of human leukocyte antigen-matched unrelated donor bone marrow: results of a matched-pair analysis. *Blood.* 2001;97(10):2957-2961.
7. Korbling M, Fliedner TM, Calvo W, Ross WM, Nothdurft W, Steinbach I. Albumin density gradient purification of canine hemopoietic blood stem cells (HBSC): long-term allogeneic engraftment without GVH-reaction. *Exp Hematol.* 1979;7(6):277-288.
8. Schmitz N, Dreger P, Suttorp M, et al. Primary transplantation of allogeneic peripheral blood progenitor cells mobilized by filgrastim (granulocyte colony-stimulating factor). *Blood.* 1995;85(6):1666-1672.
9. Wagner JE, Kernan NA, Steinbuch M, Broxmeyer HE, Gluckman E. Allogeneic sibling umbilical-cord-blood transplantation in children with malignant and non-malignant disease. *Lancet.* 1995;346(8969):214-219.
10. Laughlin MJ, Eapen M, Rubinstein P, et al. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *N Engl J Med.* 2004;351(22):2265-2275.
11. Barker JN, Weisdorf DJ, DeFor TE, et al. Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. *Blood.* 2005;105(3):1343-1347.
12. Reisner Y, Hagin D, Martelli MF. Haploidentical hematopoietic transplantation: current status and future perspectives. *Blood.* 2011;118(23):6006-6017.
13. Bach FH, Albertini RJ, Joo P, Anderson JL, Bortin MM. Bone-marrow transplantation in a patient with the Wiskott-Aldrich syndrome. *Lancet.* 1968;2(7583):1364-1366.
14. Deeg HJ, Storb R, Shulman HM, Weiden PL, Graham TC, Thomas ED. Engraftment of DLA-nonidentical unrelated canine marrow after high-dose fractionated total body irradiation. *Transplantation.* 1982;33(4):443-446.
15. Welniak LA, Blazar BR, Murphy WJ. Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu Rev Immunol.* 2007;25:139-170.
16. Pasquini MC. Why, When and Where to Report Conditioning Regimens. *Center for international blood & marrow transplant research* [Summary Slides]. 2015; https://www.cibmtr.org/Meetings/Materials/CRPDMC/Documents/2015/Conditioning_Pasquini_Tandem2015.pdf, 2018.
17. Turner BE, Collin M, Rice AM. Reduced intensity conditioning for hematopoietic stem cell transplantation: has it achieved all it set out to? *Cytotherapy.* 2010;12(4):440-454.
18. Cieri N, Mastaglio S, Oliveira G, Casucci M, Bondanza A, Bonini C. Adoptive immunotherapy with genetically modified lymphocytes in allogeneic stem cell transplantation. *Immunol Rev.* 2014;257(1):165-180.

19. Kolb HJ, Schattenberg A, Goldman JM, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood.* 1995;86(5):2041-2050.
20. Schmid C, Labopin M, Nagler A, et al. Donor lymphocyte infusion in the treatment of first hematological relapse after allogeneic stem-cell transplantation in adults with acute myeloid leukemia: a retrospective risk factors analysis and comparison with other strategies by the EBMT Acute Leukemia Working Party. *J Clin Oncol.* 2007;25(31):4938-4945.
21. Krenger W, Blazar BR, Hollander GA. Thymic T-cell development in allogeneic stem cell transplantation. *Blood.* 2011;117(25):6768-6776.
22. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood.* 1990;75(3):555-562.
23. Barrett AJ, Battiwalla M. Relapse after allogeneic stem cell transplantation. *Expert Rev Hematol.* 2010;3(4):429-441.
24. Zheng H, Matte-Martone C, Li H, et al. Effector memory CD4+ T cells mediate graft-versus-leukemia without inducing graft-versus-host disease. *Blood.* 2008;111(4):2476-2484.
25. Ringden O, Karlsson H, Olsson R, Omazic B, Uhlin M. The allogeneic graft-versus-cancer effect. *Br J Haematol.* 2009;147(5):614-633.
26. Felix NJ, Donermeyer DL, Horvath S, et al. Alloreactive T cells respond specifically to multiple distinct peptide-MHC complexes. *Nat Immunol.* 2007;8(4):388-397.
27. Baker KS, Fraser CJ. Quality of life and recovery after graft-versus-host disease. *Best Pract Res Clin Haematol.* 2008;21(2):333-341.
28. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol.* 2012;12(6):443-458.
29. Pasquini MC, Wang Z, Horowitz MM, Gale RP. 2010 report from the Center for International Blood and Marrow Transplant Research (CIBMTR): current uses and outcomes of hematopoietic cell transplants for blood and bone marrow disorders. *Clin Transpl.* 2010:87-105.
30. Billingham RE. Reactions of grafts against their hosts. *Science.* 1959;130(3381):947-953.
31. Clave E, Busson M, Douay C, et al. Acute graft-versus-host disease transiently impairs thymic output in young patients after allogeneic hematopoietic stem cell transplantation. *Blood.* 2009;113(25):6477-6484.
32. Ochs L, Shu XO, Miller J, et al. Late infections after allogeneic bone marrow transplantations: comparison of incidence in related and unrelated donor transplant recipients. *Blood.* 1995;86(10):3979-3986.
33. Blazar BR, Korngold R, Vallera DA. Recent advances in graft-versus-host disease (GVHD) prevention. *Immunol Rev.* 1997;157:79-109.
34. Krenger W, Hill GR, Ferrara JL. Cytokine cascades in acute graft-versus-host disease. *Transplantation.* 1997;64(4):553-558.
35. Kernan NA, Collins NH, Juliano L, Cartagena T, Dupont B, O'Reilly RJ. Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease. *Blood.* 1986;68(3):770-773.
36. Madrigal JA, Scott I, Arguello R, Szydlo R, Little AM, Goldman JM. Factors influencing the outcome of bone marrow transplants using unrelated donors. *Immunol Rev.* 1997;157:153-166.
37. Loiseau P, Busson M, Balere ML, et al. HLA Association with hematopoietic stem cell transplantation outcome: the number of mismatches at HLA-A, -B, -C, -DRB1, or -DQB1 is strongly associated with overall survival. *Biol Blood Marrow Transplant.* 2007;13(8):965-974.
38. Billingham RE. The biology of graft-versus-host reactions. *Harvey Lect.* 1966;62:21-78.
39. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet.* 2009;373(9674):1550-1561.
40. Filipovich AH, Weisdorf D, Pavletic S, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I.

Diagnosis and staging working group report. *Biol Blood Marrow Transplant.* 2005;11(12):945-956.
41. Vigorito AC, Campregher PV, Storer BE, et al. Evaluation of NIH consensus criteria for classification of late acute and chronic GVHD. *Blood.* 2009;114(3):702-708.
42. Vincent K, Roy DC, Perreault C. Next-generation leukemia immunotherapy. *Blood.* 2011;118(11):2951-2959.
43. Korngold R, Sprent J. Features of T cells causing H-2-restricted lethal graft-vs.-host disease across minor histocompatibility barriers. *J Exp Med.* 1982;155(3):872-883.
44. Couriel DR, Saliba RM, Giralt S, et al. Acute and chronic graft-versus-host disease after ablative and nonmyeloablative conditioning for allogeneic hematopoietic transplantation. *Biol Blood Marrow Transplant.* 2004;10(3):178-185.
45. Hill GR, Ferrara JL. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood.* 2000;95(9):2754-2759.
46. Hill GR, Krenger W, Ferrara JL. The role of cytokines in acute graft-versus-host disease. *Cytokines Cell Mol Ther.* 1997;3(4):257-266.
47. Henden AS, Hill GR. Cytokines in Graft-versus-Host Disease. *J Immunol.* 2015;194(10):4604-4612.
48. Krenger W, Ferrara JL. Graft-versus-host disease and the Th1/Th2 paradigm. *Immunol Res.* 1996;15(1):50-73.
49. Ferrara JL, Cooke KR, Pan L, Krenger W. The immunopathophysiology of acute graft-versus-host-disease. *Stem Cells.* 1996;14(5):473-489.
50. Edinger M, Hoffmann P, Ermann J, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med.* 2003;9(9):1144-1150.
51. Sakaguchi S, Ono M, Setoguchi R, et al. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev.* 2006;212:8-27.
52. Wysocki CA, Panoskaltsis-Mortari A, Blazar BR, Serody JS. Leukocyte migration and graft-versus-host disease. *Blood.* 2005;105(11):4191-4199.
53. van den Brink MR, Burakoff SJ. Cytolytic pathways in haematopoietic stem-cell transplantation. *Nat Rev Immunol.* 2002;2(4):273-281.
54. MacDonald KP, Hill GR, Blazar BR. Chronic graft-versus-host disease: biological insights from preclinical and clinical studies. *Blood.* 2017;129(1):13-21.
55. Socie G, Ritz J. Current issues in chronic graft-versus-host disease. *Blood.* 2014;124(3):374-384.
56. Hollander GA, Widmer B, Burakoff SJ. Loss of normal thymic repertoire selection and persistence of autoreactive T cells in graft vs host disease. *J Immunol.* 1994;152(4):1609-1617.
57. van den Brink MR, Moore E, Ferrara JL, Burakoff SJ. Graft-versus-host-disease-associated thymic damage results in the appearance of T cell clones with anti-host reactivity. *Transplantation.* 2000;69(3):446-449.
58. Zhao D, Young JS, Chen YH, et al. Alloimmune response results in expansion of autoreactive donor CD4+ T cells in transplants that can mediate chronic graft-versus-host disease. *J Immunol.* 2011;186(2):856-868.
59. Srinivasan M, Flynn R, Price A, et al. Donor B-cell alloantibody deposition and germinal center formation are required for the development of murine chronic GVHD and bronchiolitis obliterans. *Blood.* 2012;119(6):1570-1580.
60. Arai S, Pidala J, Pusic I, et al. A Randomized Phase II Crossover Study of Imatinib or Rituximab for Cutaneous Sclerosis after Hematopoietic Cell Transplantation. *Clin Cancer Res.* 2016;22(2):319-327.
61. Sarantopoulos S, Stevenson KE, Kim HT, et al. Altered B-cell homeostasis and excess BAFF in human chronic graft-versus-host disease. *Blood.* 2009;113(16):3865-3874.

62. Sarantopoulos S, Stevenson KE, Kim HT, et al. High levels of B-cell activating factor in patients with active chronic graft-versus-host disease. *Clin Cancer Res.* 2007;13(20):6107-6114.
63. Du J, Paz K, Flynn R, et al. Pirfenidone ameliorates murine chronic GVHD through inhibition of macrophage infiltration and TGF-beta production. *Blood.* 2017;129(18):2570-2580.
64. Baroni SS, Santillo M, Bevilacqua F, et al. Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. *N Engl J Med.* 2006;354(25):2667-2676.
65. Svegliati S, Olivieri A, Campelli N, et al. Stimulatory autoantibodies to PDGF receptor in patients with extensive chronic graft-versus-host disease. *Blood.* 2007;110(1):237-241.
66. MacDonald KP, Blazar BR, Hill GR. Cytokine mediators of chronic graft-versus-host disease. *J Clin Invest.* 2017;127(7):2452-2463.
67. Holtan SG, Pasquini M, Weisdorf DJ. Acute graft-versus-host disease: a bench-to-bedside update. *Blood.* 2014;124(3):363-373.
68. Choi SW, Reddy P. Current and emerging strategies for the prevention of graft-versus-host disease. *Nat Rev Clin Oncol.* 2014;11(9):536-547.
69. Martin PJ, Rizzo JD, Wingard JR, et al. First- and second-line systemic treatment of acute graft-versus-host disease: recommendations of the American Society of Blood and Marrow Transplantation. *Biol Blood Marrow Transplant.* 2012;18(8):1150-1163.
70. MacMillan ML, Weisdorf DJ, Wagner JE, et al. Response of 443 patients to steroids as primary therapy for acute graft-versus-host disease: comparison of grading systems. *Biol Blood Marrow Transplant.* 2002;8(7):387-394.
71. Martin PJ, Hansen JA, Buckner CD, et al. Effects of in vitro depletion of T cells in HLA-identical allogeneic marrow grafts. *Blood.* 1985;66(3):664-672.
72. Luznik L, O'Donnell PV, Fuchs EJ. Post-transplantation cyclophosphamide for tolerance induction in HLA-haploidentical bone marrow transplantation. *Semin Oncol.* 2012;39(6):683-693.
73. Kanakry CG, Ganguly S, Luznik L. Situational aldehyde dehydrogenase expression by regulatory T cells may explain the contextual duality of cyclophosphamide as both a pro-inflammatory and tolerogenic agent. *Oncoimmunology.* 2015;4(3):e974393.
74. Ho VT, Soiffer RJ. The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation. *Blood.* 2001;98(12):3192-3204.
75. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. *Nat Rev Cancer.* 2004;4(5):371-380.
76. Krenger W, Hollander GA. The immunopathology of thymic GVHD. *Semin Immunopathol.* 2008;30(4):439-456.
77. Krenger W, Hollander GA. The thymus in GVHD pathophysiology. *Best Pract Res Clin Haematol.* 2008;21(2):119-128.
78. Krenger W, Hollander GA. The role of the thymus in allogeneic hematopoietic stem cell transplantation. *Swiss Med Wkly.* 2010;140:w13051.
79. Chaudhry MS, Velardi E, Malard F, van den Brink MR. Immune Reconstitution after Allogeneic Hematopoietic Stem Cell Transplantation: Time To T Up the Thymus. *J Immunol.* 2017;198(1):40-46.
80. Roux E, Dumont-Girard F, Starobinski M, et al. Recovery of immune reactivity after T-cell-depleted bone marrow transplantation depends on thymic activity. *Blood.* 2000;96(6):2299-2303.
81. Seggewiss R, Einsele H. Immune reconstitution after allogeneic transplantation and expanding options for immunomodulation: an update. *Blood.* 2010;115(19):3861-3868.
82. Ahn H, Tay J, Shea B, et al. Effectiveness of immunoglobulin prophylaxis in reducing clinical complications of hematopoietic stem cell transplantation: a systematic review and meta-analysis. *Transfusion.* 2018.
83. Takahama Y, Ohigashi I, Baik S, Anderson G. Generation of diversity in thymic epithelial cells. *Nat Rev Immunol.* 2017;17(5):295-305.

84. Takahama Y. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol.* 2006;6(2):127-135.
85. Lind EF, Prockop SE, Porritt HE, Petrie HT. Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. *J Exp Med.* 2001;194(2):127-134.
86. Petrie HT, Zuniga-Pflucker JC. Zoned out: functional mapping of stromal signaling microenvironments in the thymus. *Annu Rev Immunol.* 2007;25:649-679.
87. Rossi FM, Corbel SY, Merzaban JS, et al. Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nat Immunol.* 2005;6(6):626-634.
88. Wurbel MA, Malissen M, Guy-Grand D, et al. Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor gammadelta(+) gut intraepithelial lymphocytes. *Blood.* 2001;98(9):2626-2632.
89. Liu C, Ueno T, Kuse S, et al. The role of CCL21 in recruitment of T-precursor cells to fetal thymi. *Blood.* 2005;105(1):31-39.
90. Love PE, Bhandoola A. Signal integration and crosstalk during thymocyte migration and emigration. *Nat Rev Immunol.* 2011;11(7):469-477.
91. Shortman K, Wu L. Early T lymphocyte progenitors. *Annu Rev Immunol.* 1996;14:29-47.
92. Allman D, Sambandam A, Kim S, et al. Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol.* 2003;4(2):168-174.
93. Godfrey DI, Kennedy J, Suda T, Zlotnik A. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J Immunol.* 1993;150(10):4244-4252.
94. Godfrey DI, Kennedy J, Mombaerts P, Tonegawa S, Zlotnik A. Onset of TCR-beta gene rearrangement and role of TCR-beta expression during CD3-CD4-CD8- thymocyte differentiation. *J Immunol.* 1994;152(10):4783-4792.
95. Bhandoola A, Sambandam A. From stem cell to T cell: one route or many? *Nat Rev Immunol.* 2006;6(2):117-126.
96. Gill J, Malin M, Sutherland J, Gray D, Hollander G, Boyd R. Thymic generation and regeneration. *Immunol Rev.* 2003;195:28-50.
97. Akashi K, Kondo M, Weissman IL. Role of interleukin-7 in T-cell development from hematopoietic stem cells. *Immunol Rev.* 1998;165:13-28.
98. Di Santo JP, Rodewald HR. In vivo roles of receptor tyrosine kinases and cytokine receptors in early thymocyte development. *Curr Opin Immunol.* 1998;10(2):196-207.
99. Zuklys S, Mayer CE, Zhanybekova S, et al. MicroRNAs control the maintenance of thymic epithelia and their competence for T lineage commitment and thymocyte selection. *J Immunol.* 2012;189(8):3894-3904.
100. Lee EN, Park JK, Lee JR, et al. Characterization of the expression of cytokeratins 5, 8, and 14 in mouse thymic epithelial cells during thymus regeneration following acute thymic involution. *Anat Cell Biol.* 2011;44(1):14-24.
101. Daley SR, Hu DY, Goodnow CC. Helios marks strongly autoreactive CD4+ T cells in two major waves of thymic deletion distinguished by induction of PD-1 or NF-kappaB. *J Exp Med.* 2013;210(2):269-285.
102. Ross EM, Bourges D, Hogan TV, Gleeson PA, van Driel IR. Helios defines T cells being driven to tolerance in the periphery and thymus. *Eur J Immunol.* 2014;44(7):2048-2058.
103. Koble C, Kyewski B. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. *J Exp Med.* 2009;206(7):1505-1513.
104. Derbinski J, Gabler J, Brors B, et al. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J Exp Med.* 2005;202(1):33-45.
105. Gillard GO, Farr AG. Features of medullary thymic epithelium implicate postnatal development in maintaining epithelial heterogeneity and tissue-restricted antigen expression. *J Immunol.* 2006;176(10):5815-5824.

106. Smith KM, Olson DC, Hirose R, Hanahan D. Pancreatic gene expression in rare cells of thymic medulla: evidence for functional contribution to T cell tolerance. *Int Immunol.* 1997;9(9):1355-1365.
107. Gardner JM, Fletcher AL, Anderson MS, Turley SJ. AIRE in the thymus and beyond. *Curr Opin Immunol.* 2009;21(6):582-589.
108. Rossi SW, Kim MY, Leibbrandt A, et al. RANK signals from CD4(+)3(-) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. *J Exp Med.* 2007;204(6):1267-1272.
109. Anderson MS, Su MA. Aire and T cell development. *Curr Opin Immunol.* 2011;23(2):198-206.
110. Liston A, Gray DH, Lesage S, et al. Gene dosage--limiting role of Aire in thymic expression, clonal deletion, and organ-specific autoimmunity. *J Exp Med.* 2004;200(8):1015-1026.
111. Klein L, Hinterberger M, Wirnsberger G, Kyewski B. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol.* 2009;9(12):833-844.
112. Ramsey C, Winqvist O, Puhakka L, et al. Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. *Hum Mol Genet.* 2002;11(4):397-409.
113. Hollander GA, Peterson P. Learning to be tolerant: how T cells keep out of trouble. *J Intern Med.* 2009;265(5):541-561.
114. Anderson MS, Venanzi ES, Klein L, et al. Projection of an immunological self shadow within the thymus by the aire protein. *Science.* 2002;298(5597):1395-1401.
115. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol.* 2005;6(4):331-337.
116. Murphy K, Weaver C. Janeway's Immunobiology, 9th Edition. *Janeway's Immunobiology, 9th Edition.* 2017:1-904.
117. Feng C, Woodside KJ, Vance BA, et al. A potential role for CD69 in thymocyte emigration. *Int Immunol.* 2002;14(6):535-544.
118. Rosen H, Goetzl EJ. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. *Nat Rev Immunol.* 2005;5(7):560-570.
119. Spiegel S, Milstien S. The outs and the ins of sphingosine-1-phosphate in immunity. *Nat Rev Immunol.* 2011;11(6):403-415.
120. Mackall CL, Granger L, Sheard MA, Cepeda R, Gress RE. T-cell regeneration after bone marrow transplantation: differential CD45 isoform expression on thymic-derived versus thymic-independent progeny. *Blood.* 1993;82(8):2585-2594.
121. Chidgey A, Dudakov J, Seach N, Boyd R. Impact of niche aging on thymic regeneration and immune reconstitution. *Semin Immunol.* 2007;19(5):331-340.
122. Pannetier C, Cochet M, Darche S, Casrouge A, Zoller M, Kourilsky P. The sizes of the CDR3 hypervariable regions of the murine T-cell receptor beta chains vary as a function of the recombined germ-line segments. *Proc Natl Acad Sci U S A.* 1993;90(9):4319-4323.
123. Gorski J, Yassai M, Zhu X, et al. Circulating T cell repertoire complexity in normal individuals and bone marrow recipients analyzed by CDR3 size spectratyping. Correlation with immune status. *J Immunol.* 1994;152(10):5109-5119.
124. Toubert A, Glauzy S, Douay C, Clave E. Thymus and immune reconstitution after allogeneic hematopoietic stem cell transplantation in humans: never say never again. *Tissue Antigens.* 2012;79(2):83-89.
125. Seemayer TA, Lapp WS, Bolande RP. Thymic epithelial injury in graft-versus-host reactions following adrenalectomy. *Am J Pathol.* 1978;93(2):325-338.
126. Beschorner WE, Hutchins GM, Elfenbein GJ, Santos GW. The thymus in patients with allogeneic bone marrow transplants. *Am J Pathol.* 1978;92(1):173-186.

127. Seddik M, Seemayer TA, Lapp WS. T cell functional defect associated with thymid epithelial cell injury induced by a graft-versus-host reaction. *Transplantation.* 1980;29(1):61-66.
128. Gartner JG. Thymic involution with loss of Hassall's corpuscles mimicking thymic dysplasia in a child with transfusion-associated graft-versus-host disease. *Pediatr Pathol.* 1991;11(3):449-456.
129. Hauri-Hohl MM, Keller MP, Gill J, et al. Donor T-cell alloreactivity against host thymic epithelium limits T-cell development after bone marrow transplantation. *Blood.* 2007;109(9):4080-4088.
130. Krenger W, Rossi S, Piali L, Hollander GA. Thymic atrophy in murine acute graft-versus-host disease is effected by impaired cell cycle progression of host pro-T and pre-T cells. *Blood.* 2000;96(1):347-354.
131. Krenger W, Rossi S, Hollander GA. Apoptosis of thymocytes during acute graft-versus-host disease is independent of glucocorticoids. *Transplantation.* 2000;69(10):2190-2193.
132. Hentschke P, Omazic B, Mattsson J, et al. T-cell receptor Vbeta repertoire after myeloablative and reduced intensity conditioning allogeneic haematopoietic stem cell transplantation. *Scand J Immunol.* 2005;61(3):285-294.
133. Fry TJ. Is a little GVHD a good thing? *Blood.* 2009;113(25):6274-6275.
134. Dertschnig S, Nusspaumer G, Ivanek R, Hauri-Hohl MM, Hollander GA, Krenger W. Epithelial cytoprotection sustains ectopic expression of tissue-restricted antigens in the thymus during murine acute GVHD. *Blood.* 2013;122(5):837-841.
135. Flowers ME, Inamoto Y, Carpenter PA, et al. Comparative analysis of risk factors for acute graft-versus-host disease and for chronic graft-versus-host disease according to National Institutes of Health consensus criteria. *Blood.* 2011;117(11):3214-3219.
136. Koyama M, Hill GR. Alloantigen presentation and graft-versus-host disease: fuel for the fire. *Blood.* 2016;127(24):2963-2970.
137. Wu T, Young JS, Johnston H, et al. Thymic damage, impaired negative selection, and development of chronic graft-versus-host disease caused by donor CD4+ and CD8+ T cells. *J Immunol.* 2013;191(1):488-499.
138. Dertschnig S, Hauri-Hohl MM, Vollmer M, Hollander GA, Krenger W. Impaired thymic expression of tissue-restricted antigens licenses the de novo generation of autoreactive CD4+ T cells in acute GVHD. *Blood.* 2015;125(17):2720-2723.
139. Parkman R. A 2-hit model for chronic GVHD. *Blood.* 2013;122(5):623-624.
140. Pavletic SZ, Fowler DH. Are we making progress in GVHD prophylaxis and treatment? *Hematology Am Soc Hematol Educ Program.* 2012;2012:251-264.
141. Rangarajan H, Yassai M, Subramanian H, et al. Emergence of T cells that recognize nonpolymorphic antigens during graft-versus- host disease. *Blood.* 2012;119(26):6354-6364.
142. Rossi S, Blazar BR, Farrell CL, et al. Keratinocyte growth factor preserves normal thymopoiesis and thymic microenvironment during experimental graft-versus-host disease. *Blood.* 2002;100(2):682-691.
143. Rossi SW, Jeker LT, Ueno T, et al. Keratinocyte growth factor (KGF) enhances postnatal T-cell development via enhancements in proliferation and function of thymic epithelial cells. *Blood.* 2007;109(9):3803-3811.
144. Brauchle M, Fassler R, Werner S. Suppression of keratinocyte growth factor expression by glucocorticoids in vitro and during wound healing. *J Invest Dermatol.* 1995;105(4):579-584.
145. Alpdogan O, Schmaltz C, Muriglan SJ, et al. Administration of interleukin-7 after allogeneic bone marrow transplantation improves immune reconstitution without aggravating graft-versus-host disease. *Blood.* 2001;98(7):2256-2265.
146. Alpdogan O, van den Brink MR. IL-7 and IL-15: therapeutic cytokines for immunodeficiency. *Trends Immunol.* 2005;26(1):56-64.

147. Mackall CL, Fry TJ, Bare C, Morgan P, Galbraith A, Gress RE. IL-7 increases both thymic-dependent and thymic-independent T-cell regeneration after bone marrow transplantation. *Blood.* 2001;97(5):1491-1497.
148. Perales MA, Goldberg JD, Yuan J, et al. Recombinant human interleukin-7 (CYT107) promotes T-cell recovery after allogeneic stem cell transplantation. *Blood.* 2012;120(24):4882-4891.
149. Dudakov JA, Hanash AM, Jenq RR, et al. Interleukin-22 drives endogenous thymic regeneration in mice. *Science.* 2012;336(6077):91-95.
150. Fukushima N, Ishii I, Contos JJ, Weiner JA, Chun J. Lysophospholipid receptors. *Annu Rev Pharmacol Toxicol.* 2001;41:507-534.
151. Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol.* 2003;4(5):397-407.
152. Spiegel S, Milstien S. Functions of the multifaceted family of sphingosine kinases and some close relatives. *J Biol Chem.* 2007;282(4):2125-2129.
153. Alvarez SE, Harikumar KB, Hait NC, et al. Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. *Nature.* 2010;465(7301):1084-1088.
154. Pappu R, Schwab SR, Cornelissen I, et al. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science.* 2007;316(5822):295-298.
155. Pham TH, Baluk P, Xu Y, et al. Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med.* 2010;207(1):17-27.
156. Chun J, Goetzl EJ, Hla T, et al. International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol Rev.* 2002;54(2):265-269.
157. Hla T, Maciag T. An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. *J Biol Chem.* 1990;265(16):9308-9313.
158. Lee MJ, Van Brocklyn JR, Thangada S, et al. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science.* 1998;279(5356):1552-1555.
159. Liu Y, Wada R, Yamashita T, et al. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Invest.* 2000;106(8):951-961.
160. Allende ML, Yamashita T, Proia RL. G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation. *Blood.* 2003;102(10):3665-3667.
161. Graeler M, Goetzl EJ. Activation-regulated expression and chemotactic function of sphingosine 1-phosphate receptors in mouse splenic T cells. *FASEB J.* 2002;16(14):1874-1878.
162. Schwab SR, Cyster JG. Finding a way out: lymphocyte egress from lymphoid organs. *Nat Immunol.* 2007;8(12):1295-1301.
163. Matloubian M, Lo CG, Cinamon G, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature.* 2004;427(6972):355-360.
164. Allende ML, Dreier JL, Mandala S, Proia RL. Expression of the sphingosine 1-phosphate receptor, S1P1, on T-cells controls thymic emigration. *J Biol Chem.* 2004;279(15):15396-15401.
165. Carlson CM, Endrizzi BT, Wu J, et al. Kruppel-like factor 2 regulates thymocyte and T-cell migration. *Nature.* 2006;442(7100):299-302.
166. Zachariah MA, Cyster JG. Neural crest-derived pericytes promote egress of mature thymocytes at the corticomedullary junction. *Science.* 2010;328(5982):1129-1135.
167. Bankovich AJ, Shiow LR, Cyster JG. CD69 suppresses sphingosine 1-phosophate receptor-1 (S1P1) function through interaction with membrane helix 4. *J Biol Chem.* 2010;285(29):22328-22337.
168. Pham TH, Okada T, Matloubian M, Lo CG, Cyster JG. S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress. *Immunity.* 2008;28(1):122-133.

169. Grigorova IL, Schwab SR, Phan TG, Pham TH, Okada T, Cyster JG. Cortical sinus probing, S1P1-dependent entry and flow-based capture of egressing T cells. *Nat Immunol.* 2009;10(1):58-65.
170. Vaessen LM, van Besouw NM, Mol WM, Ijzermans JN, Weimar W. FTY720 treatment of kidney transplant patients: a differential effect on B cells, naive T cells, memory T cells and NK cells. *Transpl Immunol.* 2006;15(4):281-288.
171. Yanagawa Y, Sugahara K, Kataoka H, Kawaguchi T, Masubuchi Y, Chiba K. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. II. FTY720 prolongs skin allograft survival by decreasing T cell infiltration into grafts but not cytokine production in vivo. *J Immunol.* 1998;160(11):5493-5499.
172. Hwang MW, Matsumori A, Furukawa Y, et al. FTY720, a new immunosuppressant, promotes long-term graft survival and inhibits the progression of graft coronary artery disease in a murine model of cardiac transplantation. *Circulation.* 1999;100(12):1322-1329.
173. Chiba K, Yanagawa Y, Masubuchi Y, et al. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. *J Immunol.* 1998;160(10):5037-5044.
174. Suzuki S, Enosawa S, Kakefuda T, et al. A novel immunosuppressant, FTY720, with a unique mechanism of action, induces long-term graft acceptance in rat and dog allotransplantation. *Transplantation.* 1996;61(2):200-205.
175. Brinkmann V, Billich A, Baumruker T, et al. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Discov.* 2010;9(11):883-897.
176. Zhang L, Wang HD, Ji XJ, Cong ZX, Zhu JH, Zhou Y. FTY720 for cancer therapy (Review). *Oncol Rep.* 2013;30(6):2571-2578.
177. Ryser MF, Ugarte F, Lehmann R, Bornhauser M, Brenner S. S1P(1) overexpression stimulates S1P-dependent chemotaxis of human CD34+ hematopoietic progenitor cells but strongly inhibits SDF-1/CXCR4-dependent migration and in vivo homing. *Mol Immunol.* 2008;46(1):166-171.
178. Smith P, O'Sullivan C, Gergely P. Sphingosine 1-Phosphate Signaling and Its Pharmacological Modulation in Allogeneic Hematopoietic Stem Cell Transplantation. *Int J Mol Sci.* 2017;18(10).
179. Taylor PA, Kelly RM, Bade ND, Smith MJ, Stefanski HE, Blazar BR. FTY720 markedly increases alloengraftment but does not eliminate host anti-donor T cells that cause graft rejection on its withdrawal. *Biol Blood Marrow Transplant.* 2012;18(9):1341-1352.
180. Walter DH, Rochwalsky U, Reinhold J, et al. Sphingosine-1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway via the S1P3 receptor. *Arterioscler Thromb Vasc Biol.* 2007;27(2):275-282.
181. Song J, Ito T, Matsuda C, et al. Inhibition of donor-derived T cells trafficking into target organs by FTY720 during acute graft-versus-host disease in small bowel transplantation. *Clin Exp Immunol.* 2006;146(1):85-92.
182. Taylor PA, Ehrhardt MJ, Lees CJ, et al. Insights into the mechanism of FTY720 and compatibility with regulatory T cells for the inhibition of graft-versus-host disease (GVHD). *Blood.* 2007;110(9):3480-3488.
183. Liu G, Burns S, Huang G, et al. The receptor S1P1 overrides regulatory T cell-mediated immune suppression through Akt-mTOR. *Nat Immunol.* 2009;10(7):769-777.
184. Obinata H, Hla T. Sphingosine 1-phosphate in coagulation and inflammation. *Semin Immunopathol.* 2012;34(1):73-91.
185. Oo ML, Thangada S, Wu MT, et al. Immunosuppressive and anti-angiogenic sphingosine 1-phosphate receptor-1 agonists induce ubiquitinylation and proteasomal degradation of the receptor. *J Biol Chem.* 2007;282(12):9082-9089.

186. Oo ML, Chang SH, Thangada S, et al. Engagement of S1P(1)-degradative mechanisms leads to vascular leak in mice. *J Clin Invest.* 2011;121(6):2290-2300.
187. Mullershausen F, Zecri F, Cetin C, Billich A, Guerini D, Seuwen K. Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors. *Nat Chem Biol.* 2009;5(6):428-434.
188. Kim YM, Sachs T, Asavaroengchai W, Bronson R, Sykes M. Graft-versus-host disease can be separated from graft-versus-lymphoma effects by control of lymphocyte trafficking with FTY720. *J Clin Invest.* 2003;111(5):659-669.
189. Orr Gandy KA, Obeid LM. Targeting the sphingosine kinase/sphingosine 1-phosphate pathway in disease: review of sphingosine kinase inhibitors. *Biochim Biophys Acta.* 2013;1831(1):157-166.
190. Kunkel GT, Maceyka M, Milstien S, Spiegel S. Targeting the sphingosine-1-phosphate axis in cancer, inflammation and beyond. *Nat Rev Drug Discov.* 2013;12(9):688-702.
191. Liu Q, Zhao X, Frissora F, et al. FTY720 demonstrates promising preclinical activity for chronic lymphocytic leukemia and lymphoblastic leukemia/lymphoma. *Blood.* 2008;111(1):275-284.
192. Liu Q, Alinari L, Chen CS, et al. FTY720 shows promising in vitro and in vivo preclinical activity by downmodulating Cyclin D1 and phospho-Akt in mantle cell lymphoma. *Clin Cancer Res.* 2010;16(12):3182-3192.
193. Pan S, Gray NS, Gao W, et al. Discovery of BAF312 (Siponimod), a Potent and Selective S1P Receptor Modulator. *ACS Med Chem Lett.* 2013;4(3):333-337.
194. Forrest M, Sun SY, Hajdu R, et al. Immune cell regulation and cardiovascular effects of sphingosine 1-phosphate receptor agonists in rodents are mediated via distinct receptor subtypes. *J Pharmacol Exp Ther.* 2004;309(2):758-768.
195. Song J, Matsuda C, Kai Y, et al. A novel sphingosine 1-phosphate receptor agonist, 2-amino-2-propanediol hydrochloride (KRP-203), regulates chronic colitis in interleukin-10 gene-deficient mice. *J Pharmacol Exp Ther.* 2008;324(1):276-283.
196. Khattar M, Deng R, Kahan BD, et al. Novel sphingosine-1-phosphate receptor modulator KRP203 combined with locally delivered regulatory T cells induces permanent acceptance of pancreatic islet allografts. *Transplantation.* 2013;95(7):919-927.
197. National Institutes of H. A two-part study to evaluate the safety, tolerability, pharmacokinetics, and efficacy of KRP203 in patients undergoing stem cell transplant for hematological malignancies. https://clinicaltrials.gov/ct2/show/NCT01830010. Accessed March 26, 2018.
198. Rivera J, Proia RL, Olivera A. The alliance of sphingosine-1-phosphate and its receptors in immunity. *Nat Rev Immunol.* 2008;8(10):753-763.
199. Rus V, Svetic A, Nguyen P, Gause WC, Via CS. Kinetics of Th1 and Th2 cytokine production during the early course of acute and chronic murine graft-versus-host disease. Regulatory role of donor CD8+ T cells. *J Immunol.* 1995;155(5):2396-2406.
200. Pickel K, Hoffmann MK. Suppressor T cells arising in mice undergoing a graft-vs-host response. *J Immunol.* 1977;118(2):653-656.
201. Kurts C, Heath WR, Carbone FR, Allison J, Miller JF, Kosaka H. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med.* 1996;184(3):923-930.
202. Barnden MJ, Allison J, Heath WR, Carbone FR. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol.* 1998;76(1):34-40.
203. Gallegos AM, Bevan MJ. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J Exp Med.* 2004;200(8):1039-1049.
204. Andorko JI, Tostanoski LH, Solano E, Mukhamedova M, Jewell CM. Intra-lymph Node Injection of Biodegradable Polymer Particles. *Jove-J Vis Exp.* 2014(83).
205. Anderson MS, Venanzi ES, Chen Z, Berzins SP, Benoist C, Mathis D. The cellular mechanism of Aire control of T cell tolerance. *Immunity.* 2005;23(2):227-239.

206. Hu Q, Nicol SA, Suen AY, Baldwin TA. Examination of thymic positive and negative selection by flow cytometry. *J Vis Exp.* 2012(68).
207. Hubert FX, Kinkel SA, Davey GM, et al. Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance. *Blood.* 2011;118(9):2462-2472.
208. Poti F, Gualtieri F, Sacchi S, et al. KRP-203, sphingosine 1-phosphate receptor type 1 agonist, ameliorates atherosclerosis in LDL-R-/- mice. *Arterioscler Thromb Vasc Biol.* 2013;33(7):1505-1512.
209. Prockop SE, Petrie HT. Regulation of thymus size by competition for stromal niches among early T cell progenitors. *J Immunol.* 2004;173(3):1604-1611.
210. Gossens K, Naus S, Corbel SY, et al. Thymic progenitor homing and lymphocyte homeostasis are linked via S1P-controlled expression of thymic P-selectin/CCL25. *J Exp Med.* 2009;206(4):761-778.
211. Yagi H, Kamba R, Chiba K, et al. Immunosuppressant FTY720 inhibits thymocyte emigration. *Eur J Immunol.* 2000;30(5):1435-1444.
212. Marabelle A, Kohrt H, Sagiv-Barfi I, et al. Depleting tumor-specific Tregs at a single site eradicates disseminated tumors. *J Clin Invest.* 2013;123(6):2447-2463.
213. Kurts C, Kosaka H, Carbone FR, Miller JF, Heath WR. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. *J Exp Med.* 1997;186(2):239-245.
214. Taniguchi RT, DeVoss JJ, Moon JJ, et al. Detection of an autoreactive T-cell population within the polyclonal repertoire that undergoes distinct autoimmune regulator (Aire)-mediated selection. *Proc Natl Acad Sci U S A.* 2012;109(20):7847-7852.
215. Pathak M, Diep PP, Lai X, Brinch L, Ruud E, Drolsum L. Ocular findings and ocular graft-versus-host disease after allogeneic stem cell transplantation without total body irradiation. *Bone Marrow Transplant.* 2018.
216. Khan IS, Mouchess ML, Zhu ML, et al. Enhancement of an anti-tumor immune response by transient blockade of central T cell tolerance. *J Exp Med.* 2014;211(5):761-768.
217. Swann JB, Smyth MJ. Immune surveillance of tumors. *J Clin Invest.* 2007;117(5):1137-1146.
218. Kimura T, Boehmler AM, Seitz G, et al. The sphingosine 1-phosphate receptor agonist FTY720 supports CXCR4-dependent migration and bone marrow homing of human CD34+ progenitor cells. *Blood.* 2004;103(12):4478-4486.
219. Juarez JG, Harun N, Thien M, et al. Sphingosine-1-phosphate facilitates trafficking of hematopoietic stem cells and their mobilization by CXCR4 antagonists in mice. *Blood.* 2012;119(3):707-716.
220. Lee RS, Kuhr CS, Sale GE, et al. FTY720 does not abrogate acute graft-versus-host disease in the dog leukocyte antigen-nonidentical unrelated canine model. *Transplantation.* 2003;76(8):1155-1158.
221. Beres AJ, Drobyski WR. The role of regulatory T cells in the biology of graft versus host disease. *Front Immunol.* 2013;4:163.
222. Lukas S, Patnaude L, Haxhinasto S, et al. No differences observed among multiple clinical S1P1 receptor agonists (functional antagonists) in S1P1 receptor down-regulation and degradation. *J Biomol Screen.* 2014;19(3):407-416.
223. Park SJ, Im DS. Sphingosine 1-Phosphate Receptor Modulators and Drug Discovery. *Biomol Ther (Seoul).* 2017;25(1):80-90.
224. Haynes L, Linton PJ, Eaton SM, Tonkonogy SL, Swain SL. Interleukin 2, but not other common gamma chain-binding cytokines, can reverse the defect in generation of CD4 effector T cells from naive T cells of aged mice. *J Exp Med.* 1999;190(7):1013-1024.
225. Dutt S, Baker J, Kohrt HE, et al. CD8+CD44(hi) but not CD4+CD44(hi) memory T cells mediate potent graft antilymphoma activity without GVHD. *Blood.* 2011;117(11):3230-3239.

226. Mehling M, Brinkmann V, Burgener AV, et al. Homing frequency of human T cells inferred from peripheral blood depletion kinetics after sphingosine-1-phosphate receptor blockade. *J Allergy Clin Immunol.* 2013;131(5):1440-1443 e1447.
227. Kaur K, Nanut MP, Ko MW, Safaie T, Kos J, Jewett A. Natural killer cells target and differentiate cancer stem-like cells/undifferentiated tumors: strategies to optimize their growth and expansion for effective cancer immunotherapy. *Curr Opin Immunol.* 2018;51:170-180.
228. Martinez RJ, Zhang N, Thomas SR, et al. Arthritogenic self-reactive CD4+ T cells acquire an FR4hiCD73hi anergic state in the presence of Foxp3+ regulatory T cells. *J Immunol.* 2012;188(1):170-181.
229. D'Souza A FC. Current Uses and Outcomes of Hematopoietic Cell Transplantation (HCT). 2017.
230. Markey KA, MacDonald KP, Hill GR. The biology of graft-versus-host disease: experimental systems instructing clinical practice. *Blood.* 2014;124(3):354-362.
231. Garnett C, Apperley JF, Pavlu J. Treatment and management of graft-versus-host disease: improving response and survival. *Ther Adv Hematol.* 2013;4(6):366-378.
232. Zeiser R, Blazar BR. Preclinical models of acute and chronic graft-versus-host disease: how predictive are they for a successful clinical translation? *Blood.* 2016;127(25):3117-3126.
233. Schroeder MA, DiPersio JF. Mouse models of graft-versus-host disease: advances and limitations. *Dis Model Mech.* 2011;4(3):318-333.
234. Schulte S, Sukhova GK, Libby P. Genetically programmed biases in Th1 and Th2 immune responses modulate atherogenesis. *Am J Pathol.* 2008;172(6):1500-1508.
235. Watanabe H, Numata K, Ito T, Takagi K, Matsukawa A. Innate immune response in Th1- and Th2-dominant mouse strains. *Shock.* 2004;22(5):460-466.
236. Reddy P, Negrin R, Hill GR. Mouse models of bone marrow transplantation. *Biol Blood Marrow Transplant.* 2008;14(1 Suppl 1):129-135.

I Abbreviations

Abbreviations for biological terms

Ab	Antibody
aGVHD	Acute graft-versus-host disease
Aire	Autoimmune regulator
ALL	Acute lymphoblastic leukemia
Allo	Allogeneic
AML	Acute myeloid leukemia
ANA	Anti-nuclear antobodies
APC	Antigen-presenting cell
APECED	Autoimmune-polyendocrinopathy-candidiasis ectodermal dystrophy
APS-1	Autoimmune polyglandular syndrome type 1
B6	"Black 6" C57BL/6 mouse strain
BAFF	B cell activating factor
BM	Bone marrow
BMT	Bone marrow transplantation
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CDR3	Complementary determining region 3
cGVHD	Chronic graft-versus-host disease
CK	Cytokeratin
CMV	Cytomegalovirus
CNI	Calcineurin inhibitors
cTEC	Cortical thymic epithelial cells
CTL	Cytotoxic T lymphocytes
CXCR	Chemokine (C-X-C motif) receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DN	CD4, CD8-Double negative
DNA	Deoxyribonucleic acid
DP	CD4, CD8-Double positive
ETP	Early thymic progenitor

Fgf7	Fibroblast growth factor 7
Flt-3 (L)	Fms-like tyrosine kinase 3 (ligand)
FoxP3	Forkhead-Box-Protein 3
FR-4	Folate receptor 4
G-CSF	Granulocyte-colony stimulating factor
GI tract	Gastrointestinal tract
GPCR	G-protein coupled receptor
Grzm B	Granzyme B
GVHD	Graft-versus-host disease
GVT	Graft-versus-tumor
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
Ig	Immunoglobulin
i.p.	Intraperitoneal
i.v.	Intravenous
ICOS	Inducible T cell co-stimulator
IFNγ	Interferon gamma
IL	Interleukin
IRBP	Interphotoreceptor retinoid-binding protein
KGF	Keratinocyte growth factor
Lin	Lineage
LN	Lymph node
LPS	Lipopolysaccharide
Luc	Luciferase
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
miHA	Minor histocompatibility antigens
mOVA	Membrane bound OVA
MPN	Myeloproliferative neoplasm
mTEC	Medullary thymic epithelial cells
mTORC1	Mammalian target of rapamycin complex 1
MTX	Methotrexate
NF-KB	Nuclear factor kappa-light-chain enhancer
NFAT	Nuclear factor of activated T cells
NHL	Non-Hodgkin lymphoma

NK cell	Natural killer cell
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PB	Peripheral blood
PD-1	Programmed cell death protein 1
pDGF	Platelet-derived growth factor
pGE	Promiscuous gene expression
pLN	Peripheral lymph node
RAG	Recombination activating gene
Reg	Regenerating islet-derived protein
RIC	Reduced intensity conditioning
RIP	Rat insulin promoter
s.c.	Subcutaneously
S1P	Sphingosine 1 phosphate
S1PR	Sphingosine 1 phosphate receptor
SC	Stem cell
Sca-1	Stem cell antigen-1
SCID	Severe combined immune deficiency
SLO	Secondary lymphoid organ
SP	CD4 or CD8-Single positive
SPF	Special pathogen-free
SPHK	Sphingosine kinase
STAT	Signal transducer and activator of transcription
TBI	Total body irradiation
TCDBM	T-cell depleted bone marrow
TCR	T-cell receptor
TEC	Thymic epithelial cell
TF	Transcription factor
TGFβ	Transforming growth factor b
T_H cell	T-helper cell
TN	CD3, CD4, CD8-triple negative
TNFα	Tumor necrosis factor alpha
TRA	Tissue restricted antigen
TRAF2	TNF receptor associated factor 2
TRAIL	TNF-related apoptosis inducing ligand
TREC	TCR rearrangement DNA excision circles

T_{reg}	Regulatory T cell
TRT	Transplant related toxicity
UEA-1	Ulex europaeus agglutinin-1
WD	Withdrawal
WT	Wild-type

Abbreviations for chemicals/ instruments/techniques

ACK	Ammonium-chloride-potassium
BrdU	5'-bromo-2'-deoxyuridin
DAPI	4', 6-iamino-2-phenylindole
dH_2O	Distilled water
EDTA	Ethylendiamintetraacetat
FACS	Fluorescence associated cell sorting
FCS	Fetal calf serum
IMDM	Iscove's modified dulbecco's medium
MACS	Magnetic associated cell sorting
MC	Methyl cellulose
NEAA	Non-essential amino acids
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RT	Room temperature
SA	Streptavidin
SN	Supernatant

Others

cGy	Centi gray
CIBMTR	Center for International Blood and Marrow Transplantation
e.g.	exempli gratia (for example)
et *al*.	et alii (and others)
FDA	Food and Drug Administration
NIH	National Institutes of Health
SD	Standard deviation

II Appendix

Manuscript:

Brief Report

TRANSPLANTATION

Impaired thymic expression of tissue-restricted antigens licenses the de novo generation of autoreactive CD4$^+$ T cells in acute GVHD

Simone Dertschnig,[1] Mathias M. Hauri-Hohl,[2] Madeleine Vollmer,[3] Georg A. Holländer,[1,4] and Werner Krenger[3]

[1]Department of Biomedicine, University of Basel and Basel University Children's Hospital, Basel, Switzerland; [2]Benaroya Research Institute, Virginia Mason Hospital, Seattle, Washington; [3]Department of Biomedicine, University Hospital Basel, Basel, Switzerland; and [4]Department of Paediatrics and the Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom

Key Points

- Loss of thymic ectopic self-antigen expression during murine acute GVHD is responsible for the de novo generation of autoreactive T cells.
- Functional impairment of the thymus medulla mechanistically links acute GVHD to posttransplantation autoimmunity.

During acute graft-versus-host disease (aGVHD) in mice, autoreactive T cells can be generated de novo in the host thymus implying an impairment in self-tolerance induction. As a possible mechanism, we have previously reported that mature medullary thymic epithelial cells (mTEC^{high}) expressing the autoimmune regulator are targets of donor T-cell alloimmunity during aGVHD. A decline in mTEC^{high} cell pool size, which purges individual tissue-restricted peripheral self-antigens (TRA) from the total thymic ectopic TRA repertoire, weakens the platform for central tolerance induction. Here we provide evidence in a transgenic mouse system using ovalbumin (OVA) as a model surrogate TRA that the de novo production of OVA-specific CD4$^+$ T cells during acute GVHD is a direct consequence of impaired thymic ectopic OVA expression in mTEC^{high} cells. Our data, therefore, indicate that a functional compromise of the medullary mTEC^{high} compartment may link alloimmunity to the development of autoimmunity during chronic GVHD. (*Blood.* 2015;125(17):2720-2723)

Introduction

Acute graft-versus-host disease (aGVHD) and chronic graft-versus-host disease (cGVHD) remain primary complications of allogeneic hematopoietic stem cell transplantation (alloHSCT).[1,2] Acute graft-versus-host disease is initiated by alloreactive donor T cells, which target a restricted set of tissues including the thymus.[3,4] Human aGVHD predisposes to cGVHD with autoimmune manifestations that are integral components of the disease.[5,6] It remains uncertain how autoimmunity is mechanistically linked to alloimmunity, but the thymus may play a role in this process.[1,4,7,8]

In the thymus, self-tolerance of the nascent T-cell receptor repertoire is attained through negative selection.[9] Essential for clonal deletion is the exposure of developing T cells to self-antigens, including those with highly restricted tissue expression. Thymic ectopic expression of tissue-restricted peripheral self-antigens (TRA) is a distinct property of mature medullary thymic epithelial cells (mTEC^{high}) that express the transcription factor autoimmune regulator (Aire).[10] Importantly, intimate associations exist between perturbations in TRA expression (independent of cause), and the susceptibility to autoimmunity in both animals and humans.[10-12]

We and others have demonstrated that mTEC^{high} are targets of donor T-cell alloimmunity during aGVHD,[3,7,13] and that thymic aGVHD interferes with the capacity of Aire$^+$mTEC^{high} to sustain TRA diversity.[14] Mechanistic links between altered thymic TRA expression and hence deviations in the TRA repertoire, the thymic production of autoreactive T-cells, and ultimately their peripheral appearance during aGVHD have not yet been established. Here we provide direct evidence in transgenic mice that de novo production of TRA-specific T-cells during aGVHD is a consequence of impaired ectopic TRA expression that results from a diminished mTEC^{high} cell pool.

Study design

Female C57BL/6 (H-2^b), Balb/c (H-2^d), CBy.PL(B6)-Thy1^a/ScrJ (Balb/c-Thy1.1;H-2^d), B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II;H-2^b), and C57BL/6-Tg (Ins2-TFRC/OVA)296Wehi/WehiJ (rat insulin promoter [RIP]-membrane-bound form of ovalbumin [mOVA];H-2^b) were purchased from the Jackson Laboratory and were kept in accordance with institutional regulations. RIP-mOVA mice express a membrane-bound form of OVA (mOVA; residues$_{139-385}$) under control of the RIP.[15] These mice express mOVA in the pancreas, but also in the thymus specifically in mTEC.[16] We bred Rag2-deficient OT-II mice, producing transgenic Vα2Vβ5 T-cell receptor (TCR) specific for OVA$_{323-339}$, with B6.SJL-Ptprc^{a}Pep3^b/BoyJ (B6.CD45.1;H-2^b)

Submitted August 22, 2014; accepted February 10, 2015. Republished online as *Blood* First Edition paper, February 17, 2015; DOI 10.1182/blood-2014-08-597245.

The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

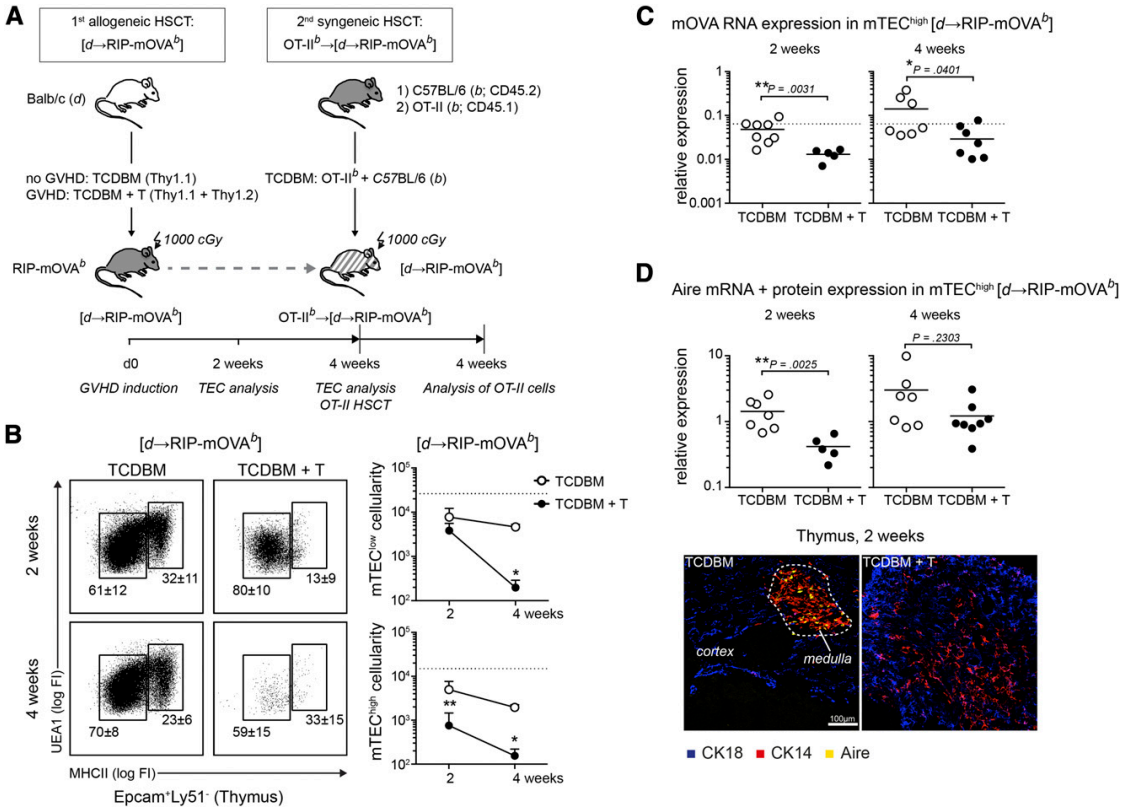


Figure 1. Acute GVHD reduces thymic ectopic expression of the surrogate self-antigen OVA in RIP-mOVA recipients. The mTEC compartment was analyzed in a transgenic murine model of H-2^d→H-2^b allo-HSCT. (A). Acute GVHD was induced in 8-week-old, lethally irradiated RIP-mOVA recipients by transfer of TCDBM mixed with Thy1.2^+ splenic T-cells from Balb/c donors (TCDBM + T group). This alloHSCT setting was designated as [*d*→RIP-mOVA^b]. As controls without aGVHD, mice received Balb/c-Thy1.1^+ TCDBM only (TCDBM group). Four weeks after the first alloHSCT, [*d*→RIP-mOVA^b] mice were lethally reirradiated and retransplanted in a second syngeneic HSCT with H-2^b TCDBM from CD45.1^+ OT-II mice mixed at a 1:4 ratio with cells from wild-type CD45.2^+ C57BL/6 mice (H-2^b). This approach generated OT-II^b→[*d*→RIP-mOVA^b] chimeric mice. (B) Flow cytometry analysis for identification of Epcam$^+$Ly51$^-$ mTEC^{low} and mTEC^{high} cells in [*d*→RIP-mOVA^b] mice in the absence (TCDBM group; ○) and presence (TCDBM + T group; ●) of aGVHD at 2 and 4 weeks after the first alloHSCT. The numbers shown in each flow cytometry dot plot represent frequencies (%, mean ± standard deviation [SD]) of the respective population among total mTEC. Line graphs depict absolute cell numbers of mTEC^{low} and mTEC^{high}. The figure represents data from 3 independent experiments with ≥3 mice per group analyzed. *$P < .05$, Mann-Whitney *U* test. (C) Expression of mOVA mRNA was determined by quantitative polymerase chain reaction in mTEC^{high}, which was purified from the total residual TEC pools isolated from mice with (●) or without (○) aGVHD at 2 and 4 weeks after the first alloHSCT. Expression is shown as relative expression normalized to GAPDH. Dashed lines indicate normal mOVA mRNA expression in naïve untransplanted RIP-mOVA mice. *$P < .05$, Mann-Whitney *U* test. (D) Expression of *Aire* mRNA was analyzed in purified mTEC^{high} cells in the alloHSCT groups above. *Aire* expression is shown as relative expression normalized to GAPDH. *$P < .05$, Mann-Whitney *U* test. To detect Aire protein, immunohistochemistry and confocal microscope analysis was performed on thymic frozen sections taken from [*d*→RIP-mOVA^b] mice with or without aGVHD (2 weeks). Cytokeratin-18 (CK18, blue) and CD14-positive cells (red) define cortical thymic epithelial cells (cTEC) and mTEC, respectively. Aire$^+$ cells are shown in yellow and localize to the thymus medulla. Thymic architecture and Aire are lost during aGVHD (lower right panel).

on a CD45.1^+ congenic background at the Benaroya Research Institute (Seattle, WA). Thymic aGVHD (H-2^d→H-2^b) was induced by transplantation of Balb/c T-cells into total body irradiated and fully major histocompatibility complex (MHC)-mismatched RIP-mOVA recipients (*d*→RIP-mOVA^b; Figure 1A; see the supplemental Methods on the *Blood* Web site). The thymic epithelial cell compartment was analyzed at 2 and 4 weeks after alloHSCT by flow cytometry (FACSAria; Becton Dickinson, Mountain View, CA). The mTECs were identified as cells with a CD45$^-$EpCam$^+$Ly51$^-$UEA1$^+$MHCII^{low} (mTEC^{low}) or MHCII^{high} (mTEC^{high}) phenotype, respectively, as described.[14] To study negative thymic selection, the *d*→RIP-mOVA^b recipients were reirradiated 4 weeks after the first alloHSCT and infused with syngeneic, rigorously (>2 log) T cell depleted OT-II bone marrow cells (TCDBM) mixed with C57BL/6 wild-type TCDBM (designated as OT-II^b→[*d*→RIP-mOVA^b]; Figure 1A). Emergence and function of OVA-specific CD4$^+$ T cells (CD45.1^+) was tested after the second syngeneic HSCT by flow cytometry (supplemental Methods). Immunohistochemistry, polymerase chain reaction, T-cell function, and statistical analyses were performed as described before[14] and in the supplement Data.

Results and discussion

We reported before that aGVHD causes a quantitative decline in the Aire$^+$ mTEC^{high} pool and consequently a less diverse TRA repertoire, thus impairing the molecular platform for central tolerance induction.[14] It remained uncertain, however, whether such mechanism sufficed for the escape of TRA-specific TCR from thymic deletion. Because the precise antigen specificities of autoreactive effector T cells in cGVHD remain unidentified,[17] we used mOVA as a surrogate self-antigen and tested whether loss of mOVA expression affected central deletion of OVA-specific T cells during aGVHD. We chose the OT-II→RIP-mOVA system because (1) thymic mOVA expression is restricted to mTEC[16]; (2) TCR selection against mOVA recapitulates physiological tolerance induction to TRA in the thymus medulla[16,18-21]; and (3) a reduction of

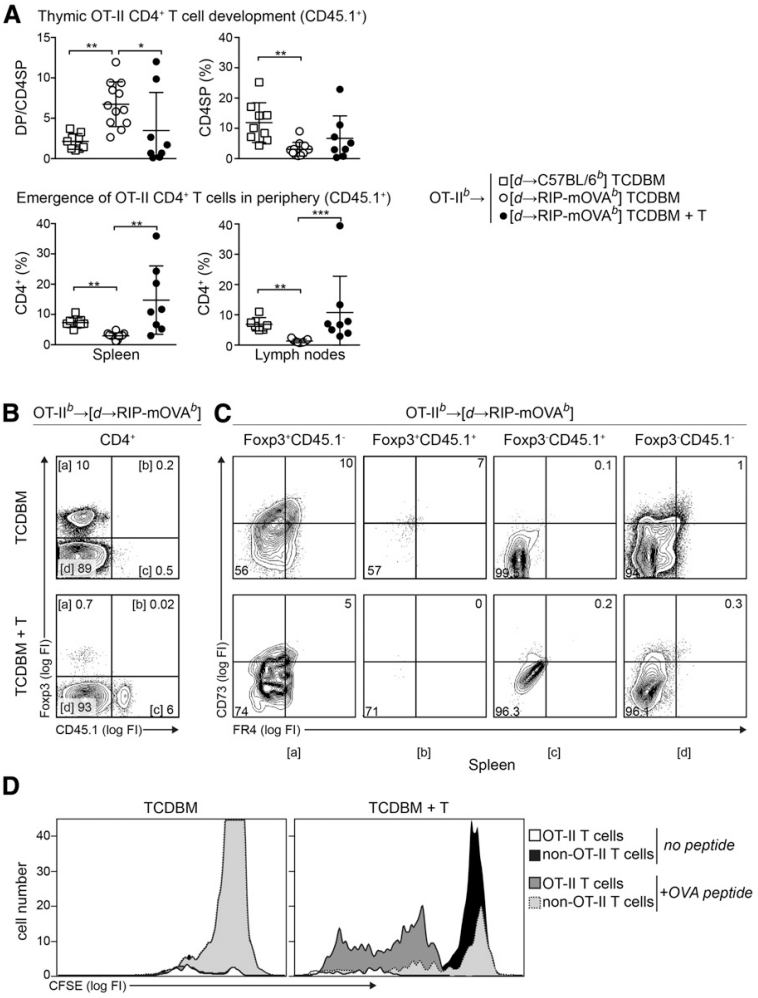


Figure 2. OVA-specific T-cell clones escape negative selection during aGVHD. Four weeks after their first alloHSCT, the [d→RIP-mOVA^b] mice with (●) or without (○) aGVHD received TCDBM (H-2^b) from CD45.1$^+$ OT-II and CD45.2$^+$ C57BL/6 mice in a second syngeneic HSCT as described in Figure 1A. A third group included a second syngeneic HSCT into nontransgenic GVHD$^-$ recipients of a first alloHSCT (□ TCDBM OT-II^b→[d→C57BL/6^b]). OT-II CD4$^+$ T-cells were analyzed in primary and secondary lymphoid organs 4 weeks later in all 3 groups. (A) Upper panels: Thymic OT-II CD4$^+$ T-cell development. Top left: the DP/CD4SP ratios between immature and mature thymocytes derived from CD45.1$^+$ OT-II bone marrow-derived cells were calculated and are shown as mean ± SD. The figure represents data from 3 independent experiments. *P < .05, Kruskall-Wallis test with Dunn's multiple comparison test. Top right: Flow cytometric analysis of CD4SP thymocytes (live gate defined by 4,6 diamidino-2-phenylindole$^-$ cells). The frequencies of CD45.1$^+$ OT-II cells among total thymic CD4SP cells are shown as mean ± SD. Lower panels: Emergence of OT-II cells in the periphery. The frequencies of OT-II cells (CD45.1$^+$CD4$^+$) among total CD4$^+$ T cells in the spleens and lymph nodes are shown as mean ± SD. The figure represents combined data from 3 independent experiments with ≥6 mice analyzed per group. *P < .05, Kruskall-Wallis test with Dunn's multiple comparison test. (B) Intracellular Foxp3 expression was analyzed in splenic CD4$^+$ T cells isolated from OT-II^b→[d→RIP-mOVA^b] mice with or without aGVHD at 4 weeks after the second syngeneic HSCT. Flow cytometry plots depict surface CD45.1 and intracellular Foxp3 expression. (C) Quadrants [a], [b], [c], and [d] were further analyzed for surface expression of folate receptor 4 (FR4) and CD73. Data are representative of at least 2 independent experiments with ≥6 mice analyzed per group. (D) Cultures of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4$^+$ T-cells isolated from spleens and lymph nodes of transplanted mice were used to detect ex vivo the proliferative response to OVA$_{323\text{-}339}$ peptide presented by syngeneic APC (see supplemental Methods). Histograms of CFSE fluorescence in CD4$^+$ responder cells are shown (log fluorescence intensity and cell numbers). Data are representative for ≥6 mice analyzed per group. The data substantiate that peripheral OT-II cells are responsive to their cognate antigen and therefore do not enter into an anergic state.

mOVA mRNA in mTEC by <30% suffices for RIP-mOVA thymi to fail to delete OT-II cells.[22]

We studied aGVHD in lethally irradiated RIP-mOVA recipients of fully MHC-mismatched Balb/c donors (designated [d→RIP-mOVA^b]; Figures 1A and supplemental Figure 1). Consistent with previous data that reduction in mTEC compartment size is a universal manifestation of thymic aGVHD,[14] total mTEC^{low}, and mTEC^{high}, cells were diminished in numbers to ≤10^3 cells/mouse at 4 weeks after alloHSCT (Figure 1B). In addition, the presence of thymic aGVHD in [d→RIP-mOVA^b] mice (supplemental Figure 1) reduced global OVA mRNA levels in total residual mTEC^{high} cell pools isolated after transplantation (Figure 1C). Our data also consistently demonstrated a reduction in the expression of both *Aire* mRNA and protein as a consequence of aGVHD-mediated TEC injury (Figure 1D). Because Aire regulates OVA expression[19] and because the Aire$^+$mTEC^{high} subset is reduced in numbers during aGVHD,[14] our data argues that loss of Aire$^+$mTEC^{high} was responsible for the deficiency in thymic OVA during aGVHD.

We postulated that aGVHD interfered with negative selection of the OVA TCR because (1) *Aire*$^{-/-}$RIP-mOVA mice cannot efficiently delete OT-II T-cells[19] and (2) total thymic mOVA expression levels correlate with deletion efficacy of OVA-reactive TCR.[16,18,19,21,22] To test our hypothesis, transgenic recipients with or without aGVHD were reirradiated and transplanted with syngeneic OT-II TCDBM (designated as OT-II^b→[d→RIP-mOVA^b]; Figure 1A). Thymic OT-II CD4$^+$ T-cell development was monitored by assessment of CD45.1$^+$ cells. An adequate ratio (7:1)[16,21] between CD45.1$^+$ immature CD4$^+$8$^+$ (DP) and mature CD4$^+$CD8$^-$ thymocytes (CD4SP) indicated regular deletion of OVA-specific TCR in OT-II^b→[d→RIP-mOVA] mice without disease, as expected (Figure 2A, top left). Much lower DP/CD4SP ratios were observed in transgenic recipients with aGVHD (low thymic mOVA), indicating inefficient deletion of OT-II cells. DP/CD4SP ratios were in the majority of these mice not distinguishable from ratios in OT-II^b→[d→C57BL/6] nondeleting controls (no thymic mOVA). Deficient elimination of OT-II cells in transgenic mice with aGVHD was substantiated by twofold to threefold higher frequencies of CD45.1$^+$CD4SP among total thymic CD4SP cells when compared with mice without aGVHD (Figure 2A, top right; supplemental Figure 2). Thus, an aGVHD-mediated loss of OVA expression in

mTEC^{high} resulted in an unopposed escape of "forbidden" OVA-specific Vα2$^+$Vβ5$^+$CD4$^+$ T-cell clones (Barnden et al.[23]; supplemental Figure 2) within the host thymus. OT-II cells were also present in the lymph nodes and spleens of transgenic mice with aGVHD (Figure 2A, bottom). Because mature OT-II T-cells were not passively transferred from donor grafts (supplemental Figure 2), formation of the peripheral OT-II pool was thymus-dependent.

In transgenic recipients with aGVHD, the fraction of C57BL/6 (CD45.1$^-$) donor bone marrow–derived Foxp3$^+$ regulatory T-cells (T_{reg}) among total splenic CD4$^+$ cells were reduced in frequency from a normal average of 10% to an average $<$1% (Figure 2B, upper left quadrants [a]). Among Foxp3$^+$CD45.1$^-$ cells, some were FR4^{high}CD73^{high}, documenting their anergic phenotype[24] (Figure 2C, far left panels [a]). In contrast, emerging OT-II (CD45.1$^+$) cells were exclusively Foxp3$^-$ conventional T-cells whose FR4$^-$CD73$^-$ phenotype suggested that they were nonanergic[24] (Figure 2C, panels [c]). Indeed, CD45.1$^+$CD4$^+$ (OT-II) cells, but not CD45.1$^-$CD4$^+$ (non-OT-II) cells, isolated from aGVHD mice vigorously responded to OVA peptide in culture (Figure 2D).

Taken together, we provide direct evidence in transgenic mice using OVA as model TRA that intrathymic de novo production of TRA-specific CD4$^+$ T-cells during aGVHD is triggered by impaired ectopic TRA expression. These OVA-reactive T cells are exported into a periphery that is characterized by T_{reg} deficiency. We advocate that functional compromise of the mTEC compartment may provide a pathogenic link between alloimmunity and the development of autoimmunity.[25] The identification of the specificities of autoreactive effector T cells in cGVHD will allow to test whether such a mechanism operates not only for a surrogate TRA, but is universal for thymic ectopic expression of those TRA that are present in tissues known to be targets of cGVHD.

Acknowledgments

The authors thank Dr Gabor Szinnai (University Children's Hospital, Basel) for his constructive review of our manuscript, Katrin Hafen (Basel) for expert technical help, and Nicole von Burg (Basel) for providing OVA peptide.

This work was supported by Swiss National Science Foundation (grants 310030-129838 [W.K.] and 310010-122558 [G.A.H.]), and by a grant from the Hematology Research Foundation, Basel, Switzerland (W.K.).

Authorship

Contribution: S.D. and M.H.H. designed and performed the study; M.V. performed the study; W.K. and G.A.H. shared senior authorship; W.K. and G.A.H. designed the work; and S.D. and W.K. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Simone Dertschnig, Department of Biomedicine, University of Basel, Mattenstrasse 28, 4058 Basel, Switzerland; e-mail: simone.dertschnig@unibas.ch.

References

1. Socié G, Ritz J. Current issues in chronic graft-versus-host disease. *Blood.* 2014;124(3):374-384.
2. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol.* 2012;12(6):443-458.
3. Na IK, Lu SX, Yim NL, et al. The cytolytic molecules Fas ligand and TRAIL are required for murine thymic graft-versus-host disease. *J Clin Invest.* 2010;120(1):343-356.
4. Krenger W, Blazar BR, Holländer GA. Thymic T-cell development in allogeneic stem cell transplantation. *Blood.* 2011;117(25):6768-6776.
5. Pavletic SZ, Fowler DH. Are we making progress in GVHD prophylaxis and treatment? *Hematology Am Soc Hematol Educ Program.* 2012;2012:251-264.
6. Flowers ME, Inamoto Y, Carpenter PA, et al. Comparative analysis of risk factors for acute graft-versus-host disease and for chronic graft-versus-host disease according to National Institutes of Health consensus criteria. *Blood.* 2011;117(11):3214-3219.
7. Wu T, Young JS, Johnston H, et al. Thymic damage, impaired negative selection, and development of chronic graft-versus-host disease caused by donor CD4$^+$ and CD8$^+$ T cells. *J Immunol.* 2013;191(1):488-499.
8. Teshima T, Reddy P, Liu C, Williams D, Cooke KR, Ferrara JL. Impaired thymic negative selection causes autoimmune graft-versus-host disease. *Blood.* 2003;102(2):429-435.
9. Vicente R, Swainson L, Marty-Grès S, et al. Molecular and cellular basis of T cell lineage commitment. *Semin Immunol.* 2010;22(5):270-275.
10. Anderson MS, Su MA. Aire and T cell development. *Curr Opin Immunol.* 2011;23(2):198-206.
11. Liston A, Gray DH, Lesage S, et al. Gene dosage—limiting role of Aire in thymic expression, clonal deletion, and organ-specific autoimmunity. *J Exp Med.* 2004;200(8):1015-1026.
12. Klein L, Hinterberger M, Wirnsberger G, Kyewski B. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol.* 2009;9(12):833-844.
13. Hauri-Hohl MM, Keller MP, Gill J, et al. Donor T-cell alloreactivity against host thymic epithelium limits T-cell development after bone marrow transplantation. *Blood.* 2007;109(9):4080-4088.
14. Dertschnig S, Nusspaumer G, Ivanek R, Hauri-Hohl MM, Holländer GA, Krenger W. Epithelial cytoprotection sustains ectopic expression of tissue-restricted antigens in the thymus during murine acute GVHD. *Blood.* 2013;122(5):837-841.
15. Kurts C, Heath WR, Carbone FR, Allison J, Miller JF, Kosaka H. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med.* 1996;184(3):923-930.
16. Gallegos AM, Bevan MJ. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J Exp Med.* 2004;200(8):1039-1049.
17. Hess AD. Equal opportunity targeting in chronic GVHD. *Blood.* 2012;119(26):6183-6184.
18. Derbinski J, Schulte A, Kyewski B, Klein L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol.* 2001;2(11):1032-1039.
19. Anderson MS, Venanzi ES, Chen Z, Berzins SP, Benoist C, Mathis D. The cellular mechanism of Aire control of T cell tolerance. *Immunity.* 2005;23(2):227-239.
20. Suen AY, Baldwin TA. Proapoptotic protein Bim is differentially required during thymic clonal deletion to ubiquitous versus tissue-restricted antigens. *Proc Natl Acad Sci USA.* 2012;109(3):893-898.
21. Hu Q, Nicol SA, Suen AY, Baldwin TA. Examination of thymic positive and negative selection by flow cytometry. *J Vis Exp.* 2012;(68):4269.
22. Hubert FX, Kinkel SA, Davey GM, et al. Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance. *Blood.* 2011;118(9):2462-2472.
23. Barnden MJ, Allison J, Heath WR, Carbone FR. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol.* 1998;76(1):34-40.
24. Martinez RJ, Zhang N, Thomas SR, et al. Arthritogenic self-reactive CD4$^+$ T cells acquire an FR4^{hi}CD73^{hi} anergic state in the presence of Foxp3$^+$ regulatory T cells. *J Immunol.* 2012;188(1):170-181.
25. Parkman R. A 2-hit model for chronic GVHD. *Blood.* 2013;122(5):623-624.

Comment

trigger the same response to generate intracellular ROS. Recent studies have shown that CD36 signaling in macrophages is quite complex and often requires cooperation with other membrane proteins, including toll-like receptors,[10] tetraspanins, integrins, and the sodium-potassium ATPase. Whether these CD36 partners are involved in ROS generation and whether different DAMPs generate differential downstream signals based on their capacity to recruit specific CD36 membrane partners remains to be determined, as do the mechanisms by which ROS target the cGMP signaling pathway. This interesting paper, however, points to potential new targets for lowering thrombotic risk in highly susceptible patient populations.

Conflict-of-interest disclosure: The author declares no competing financial interests. ■

REFERENCES

1. Magwenzi S, Woodward C, Wraith KS, et al. Oxidized LDL activates blood platelets through CD36/NOX2–mediated inhibition of the cGMP/protein kinase G signaling cascade. *Blood.* 2015;125(17):2693-2703.
2. Podrez EA, Byzova TV, Febbraio M, et al. Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype. *Nat Med.* 2007;13(9):1086-1095.
3. Zhu W, Li W, Silverstein RL. Advanced glycation end products induce a prothrombotic phenotype in mice via interaction with platelet CD36. *Blood.* 2012;119(25):6136-6144.
4. Ghosh A, Li W, Febbraio M, et al. Platelet CD36 mediates interactions with endothelial cell-derived microparticles and contributes to thrombosis in mice. *J Clin Invest.* 2008;118(5):1934-1943.
5. Wang Y, Fang C, Gao H, et al. Platelet-derived S100 family member myeloid-related protein-14 regulates thrombosis. *J Clin Invest.* 2014;124(5):2160-2171.
6. Silverstein RL, Li W, Park YM, Rahaman SO. Mechanisms of cell signaling by the scavenger receptor CD36: implications in atherosclerosis and thrombosis. *Trans Am Clin Climatol Assoc.* 2010;121:206-220.
7. Nergiz-Unal R, Lamers MM, Van Kruchten R, et al. Signaling role of CD36 in platelet activation and thrombus formation on immobilized thrombospondin or oxidized low-density lipoprotein. *J Thromb Haemost.* 2011;9(9):1835-1846.
8. Wraith KS, Magwenzi S, Aburima A, Wen Y, Leake D, Naseem KM. Oxidized low-density lipoproteins induce rapid platelet activation and shape change through tyrosine kinase and Rho kinase-signaling pathways. *Blood.* 2013;122(4):580-589.
9. Li W, Febbraio M, Reddy SP, et al. CD36 participates in a signaling pathway that regulates ROS formation in murine VSMCs. *J Clin Invest.* 2010;120(11):3996-4006.
10. Stewart CR, Stuart LM, Wilkinson K, et al. CD36 ligands promote sterile inflammation through assembly of a toll-like receptor 4 and 6 heterodimer. *Nat Immunol.* 2010;11(2):155-161.

● ● ● TRANSPLANTATION

Comment on Dertschnig et al, page 2720

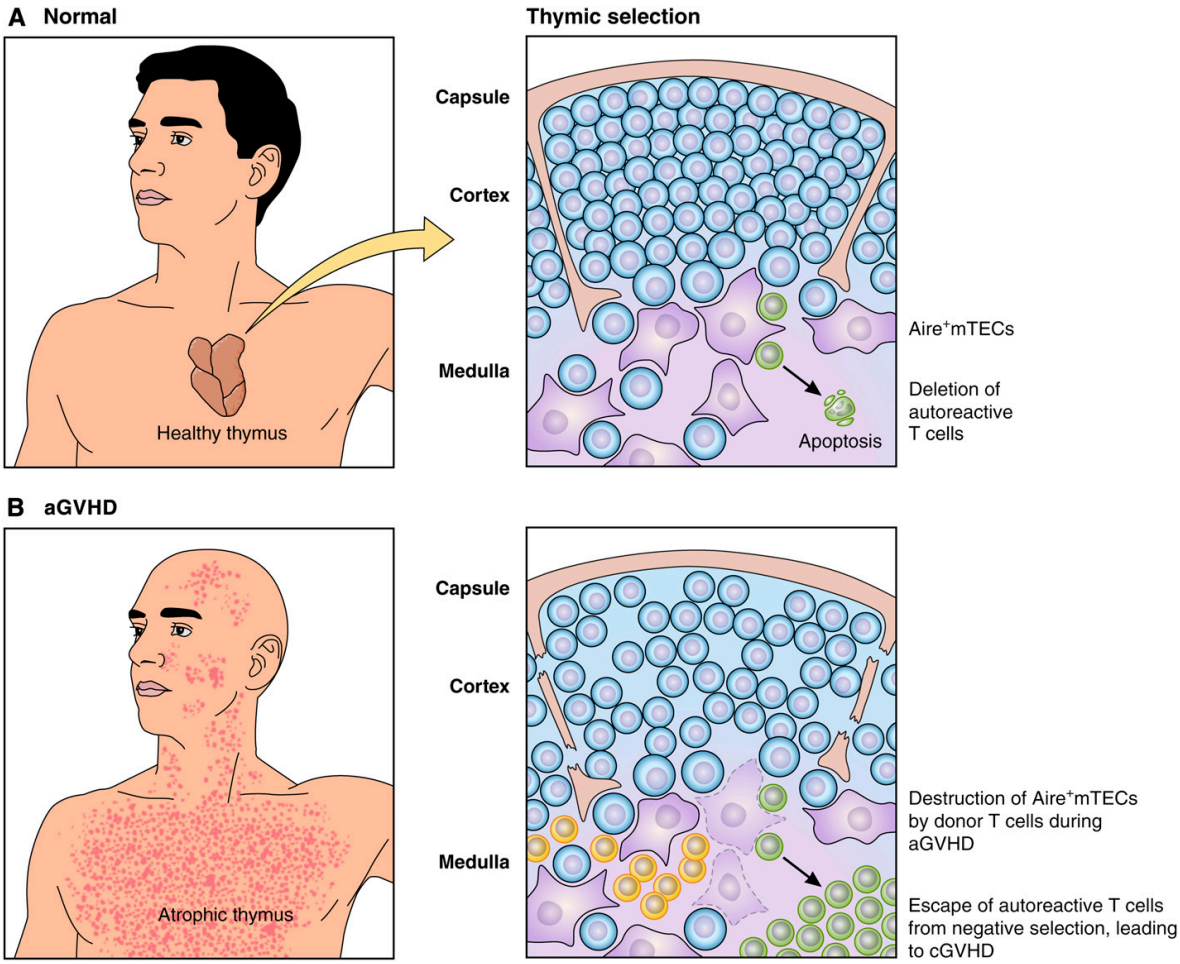
GVHD clears the Aire in thymic selection

Mojibade N. Hassan and Edmund K. Waller WINSHIP CANCER INSTITUTE EMORY UNIVERSITY

In this issue of *Blood*, Dertschnig et al describe the development of autoreactive T cells from the thymus in mice that had previously developed acute graft-versus-host-disease (aGVHD).[1]

The findings of Dertschnig et al provide an important mechanistic link between the pathogenesis of acute graft-versus-host disease (aGVHD) and its more indolent cousin, chronic (c)GVHD.[2,3] aGVHD typically occurs in the first 100 days after allogeneic hematopoietic stem cell transplant (HSCT), and is mediated by mature T cells present in the donor graft that cause local inflammation and damage epithelial cells in the skin, liver, and gastrointestinal tract.[2] In contrast, cGVHD typically develops 4 to 6 months posttransplant due to antigen-specific donor immune cells that cause autoimmune clinical manifestations including sclerosis and fibrosis in tissues and organs.[3] After donor stem cells have engrafted in the bone marrow, including the setting of major histocompatibility complex (MHC) mismatched donor and recipient,[4] donor-derived T cells developing in the recipient thymus should undergo negative selection, one aspect of central tolerance, to eliminate autoreactive clones. Thus, the presence of autoreactive donor-derived T cells in the periphery that recognize self-peptides in patients with cGVHD represents a failure of negative selection. Although aGVHD is well established as a risk factor for the development of cGVHD,[5] the mechanism for the association has not been clear. A clue to understanding the relationship between acute and chronic GVHD is based on the normal process by which autoreactive T cells are eliminated. Activity of the *Aire* gene in the thymus leads to low levels of synthesis of a smorgasbord of tissue-restricted proteins and subsequent presentation of peptides derived from these proteins on medullary thymic epithelial cells (mTECs). During physiological negative selection, thymocytes that are autoreactive to proteins expressed in peripheral organs are eliminated when they come into contact with mTECs expressing peptides normally restricted to peripheral tissues. The study by Dertschnig et al provides important insight into how negative selection fails in the setting of allogeneic transplant, and connects the pathophysiology of aGVHD to the subsequent development of cGVHD.[1]

The authors use a RIP-mOVA mouse model system, in which ovalbumin (OVA) is expressed under the control of the tissue-specific rat insulin promoter (RIP), as a model for a tissue-specific protein that should cause negative selection in the thymus. Using RIP-OVA transgenic mice as transplant recipients, membrane-bound (m)OVA is expressed in pancreatic islets and by $Aire^+$mTECs, normally leading to thymic elimination of autoreactive T cells that recognize mOVA peptides. The authors established that alloreactive T cells that are present in a donor graft from an MHC-mismatched mouse strain cause destruction and elimination of $Aire^+$mTECs during aGVHD. Mice that had developed aGVHD and lacked $Aire^+$mTECs were then retransplanted with congenic T cell–depleted bone marrow from MHC matched OT-II donor mice that express a T-cell receptor on $CD4^+$ T cells specific to an mOVA peptide. The authors show that when negative selection is intact in control mice that did not develop aGVHD, transgenic OT-II T cells are deleted from the repertoire during intrathymic T-cell development. In mice with a history of aGVHD and that lack $Aire^+$mTECs expressing mOVA, OT-II T cells survived negative selection and migrated to the periphery unchecked. The findings of Dertschnig et al illustrate the relationship between aGVHD and the failure of central tolerance: autoreactive T cells were generated de novo following the second



Alloreactive donor T cells destroy Aire$^+$mTECs during aGVHD, allowing escape of autoreactive T cells that contribute to cGVHD. (A) In healthy individuals, mTECs (purple) mediate negative selection of thymocytes (blue) to eliminate autoreactive T cells (green) recognizing self-peptides presented on MHC molecules. (B) During aGVHD, alloreactive donor T cells (yellow) damage the thymus and eliminate mTECs, allowing escape of autoreactive T cells (green) that contribute to the pathogenesis of cGVHD in the periphery. Professional illustration by Patrick Lane, ScEYEnce Studios.

MHC-matched transplant of T cell–depleted OT-II bone marrow due to loss of mTECs in the damaged thymus (see figure).

Dertschnig et al show that the mOVA-specific T cells generated in their system are highly reactive to mOVA peptide in culture but leave unanswered the important question of whether these mOVA-specific T cells are generated in sufficient number and with the functional ability to cause autoimmunity in these mice. Because mOVA is under the RIP promoter in the transgenic mice used in this model, this question could be answered by assessing damage to pancreatic islet cells and ensuing Type 1 diabetes. Findings pertaining to this question have the potential to further the understanding of the link between cGVHD and the graft-versus-leukemia (GVL) effect of the allogeneic transplant. Because the development of cGVHD is also associated with a reduced risk of leukemia relapse and increased GVL activity, it may be that leukemia-associated antigens are also ectopically expressed by Aire$^+$mTECs. If so, elimination of mTECs may permit the survival of GVL-specific donor-derived T cells generated de novo in the recipient thymus. However, the contribution of the thymus to the GVL effect may be limited by decreased thymic T-cell output due to atrophy and depopulation and thinning of the thymic cortex that follows the development of aGVHD (see figure).[6]

With the findings from Dertschnig et al in hand, what methods can be used to eliminate, reduce, or alter function of alloreactive T cells in donor hematopoietic stem cell grafts? Although outright elimination of all alloreactive effector T cells in allogeneic HSCT has had limited success due to increased risks of leukemia relapse and delayed immune reconstitution, a promising new approach is to use recipient cells to condition donor grafts to generate antigen-specific regulatory T (T_{reg}) cells that limit GVHD and tissue damage when transplanted in combination with alloreactive T cells.[7] Such an approach might

be of great value if the immune-dominant peptides that are the targets of donor T cells that mediate cGVHD attack of the skin, liver, and lungs might be used to generate T_{reg} cells that limit cGVHD while sparing cytotoxic effector cells that mediate GVL. It is also of interest to determine whether ex vivo–generated donor T_{reg} cells could be used to prevent the damage to the thymus during aGVHD and reduce the incidence of subsequent cGVHD.

Dertschnig et al eloquently demonstrate that destruction of $Aire^+$mTECs during aGVHD leads to de novo generation of inappropriately licensed autoreactive T cells and has helped "clear the Aire" regarding the pathophysiology of acute and chronic GVHD.

Conflict-of-interest disclosure: The authors declare no conflicting financial interests. ■

REFERENCES

1. Dertschnig S, Hauri-Hohl MM, Vollmer M, Holländer GA, Krenger W. Impaired thymic expression of tissue-restricted antigens licenses the de novo generation of autoreactive $CD4^+$ T cells during murine acute GVHD. *Blood*. 2015;125(17):2720-2723.

2. Ball LM, Egeler RM; EBMT Paediatric Working Party. Acute GvHD: pathogenesis and classification. *Bone Marrow Transplant*. 2008; 41(Suppl 2):S58-S64.

3. Lee S, Flowers M. Recognizing and managing chronic graft-versus-host disease. *Hematology Am Soc Hematol Educ Program*. 2008;2008: 134-141.

4. Shizuru JA, Weissman IL, Kernoff R, Masek M, Scheffold YC. Purified hematopoietic stem cell grafts induce tolerance to alloantigens and can mediate positive and negative T cell selection. *Proc Natl Acad Sci USA*. 2000;97(17):9555-9560.

5. Remberger M, Kumlien G, Aschan J, et al. Risk factors for moderate-to-severe chronic graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2002; 8(12):674-682.

6. Weinberg K, Blazar BR, Wagner JE, et al. Factors affecting thymic function after allogeneic hematopoietic stem cell transplantation. *Blood*. 2001; 97(5):1458-1466.

7. Davies JK, Nadler LM, Guinan EC. Expansion of allospecific regulatory T cells after anergized, mismatched bone marrow transplantation. *Sci Transl Med*. 2009;1(1): 1ra3.

Supplementary figures

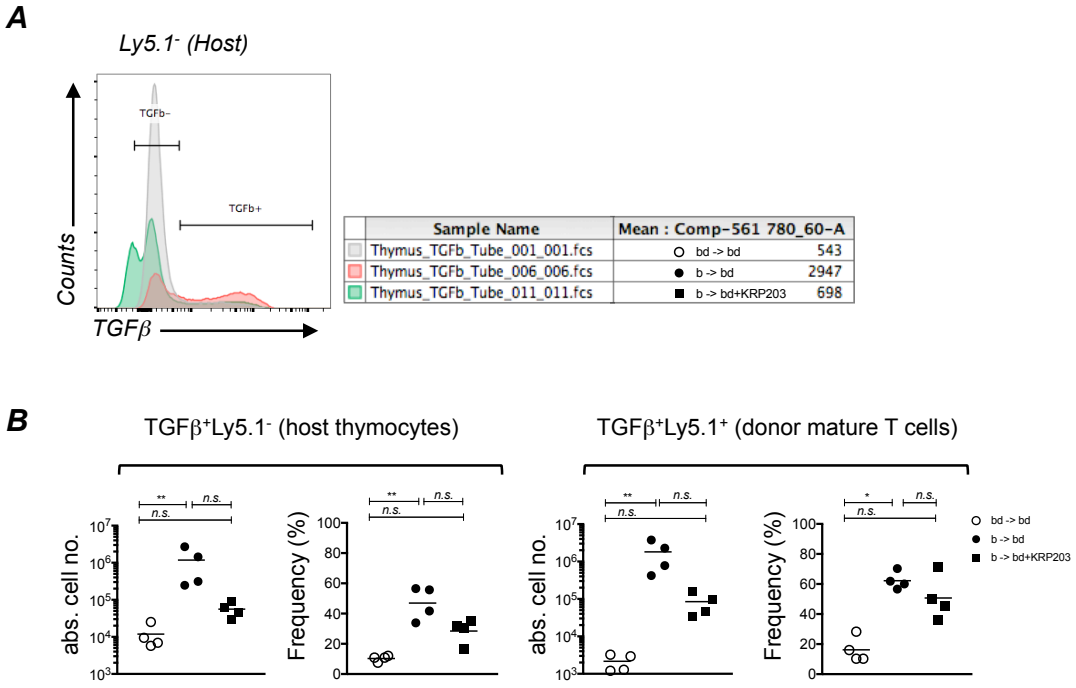


Figure S1: ***Pro-inflammatory cytokines are reduced in the thymus by KRP203 in the presence of aGVHD.*** *Acute GVHD (aGVHD) (b→bd) was induced in 8-week old female BDF1 recipients by injection of splenic T cells from B6 donors. The mean fluorescence intensity (MFI) of TGFβ^{+} cells was determined 2 weeks after T-cell transfer by flow cytometry (A). Absolute cell numbers and frequencies of TGFβ-secreting recipient T cells (Ly5.1^{-}) (left panel) and donor T cells (CD45.1^{+}) (right panel) were analyzed in mice without aGVHD (○), mice that developed aGVHD (●) and mice that received T cells and KRP203 (3mg/kg, i.p., every 2nd day from day-1 until the end of experiment) (■). *p< 0.05, Kruskal-Wallis and Dunn's multiple comparison test.*

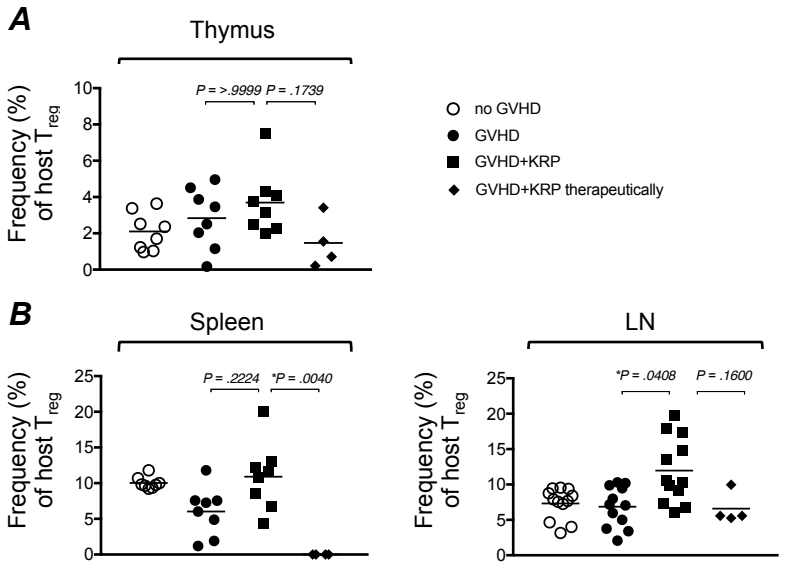


Figure S2: ***T_{reg} numbers are increased upon prophylactic KRP203 administration.*** *Acute GVHD (b→bd) was induced in 8-week old female BDF1 recipients by injection of splenic T cells from B6 donors. Intracelullar FoxP3 expression was analyzed and frequencies of host T_{regs} ($CD4^+CD25^+FoxP3^+$) were determined 2 weeks after T-cell transfer in mice without GVHD (O), with GVHD (●), with GVHD and prophylactic (start day-1) KRP203 administration (■) and therapeutic application (start day 7) (◆) (3mg/kg i.p. every 2nd day) in the thymus (A) and the SLO such as spleen (left graph) and LN (right graph) (B). This figure represents data from 3 independent experiments with ≤ 3 mice per group. *p< 0.05, Kruskal-Wallis and Dunn's multiple comparison test.*

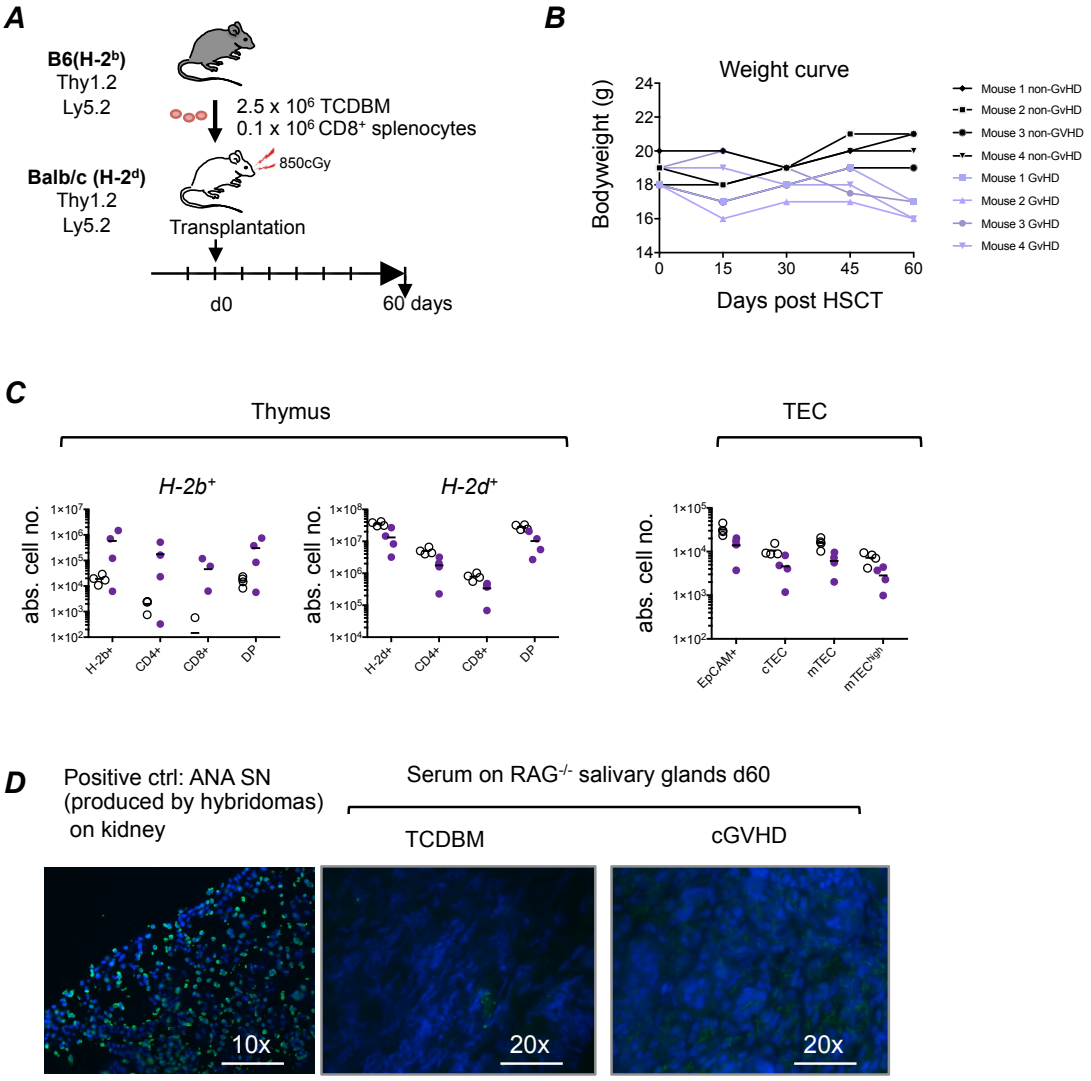


Figure S3: ***Establishment of cGVHD model.*** *Chronic GVHD (cGVHD) (b→d) was induced in a fully MHC-mismatched murine allo-HSCT model (H-2^b→H-2^d) in lethally irradiated Balb/c recipients by co-injection of TCDBM + 0.1*10^6 CD8$^+$T cells (cGVHD (●)), TCDBM + T cells+ KRP203 treatment (■) or TCDBM alone (○) from B6 mice that served as a control. Mice were analyzed at day 60 post-HSCT (A). Weight curve of mice with and without GVHD (B). Absolute cell numbers of H-2b$^+$ cells (donor) and H-2d$^+$ (recipient) at day 60 post allo-HSCT (two graphs left) and absolute cell numbers of thymic epithelial cell (TEC) compartment from the host (C). Measurement of auto-Ab. Supernatant of anti-nuclear antobodies (ANA) served as a control and was detected on kidney (left picture). After day 60, serum was taken from mice with and without cGVHD and tested on salivary glands (pictures on the right) (D).*

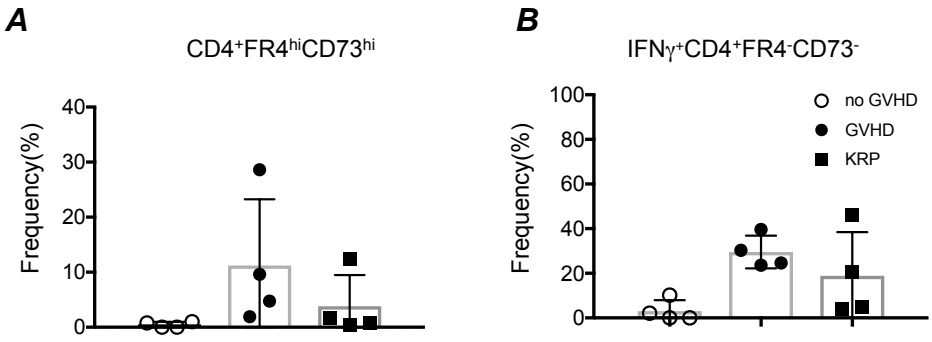


Figure S4: ***T cells do not become anergic after KRP203 administration.*** *Acute GVHD (b→bd) was induced in 8-week old female BDF1 recipients by injection of splenic T cells from B6 donors. Mice were sacrificed at 2 weeks and lymphocytes were isolated from lymph nodes. Anergic cells were determined by the surface marker CD73 and folate receptor 4 (FR-4) in mice without GVHD (O), with GVHD (●) and with GVHD and prophylactic (start day-1) KRP203 administration (3mg/kg i.p. every 2nd day) (A). IFNγ cytokine secretion was measured by flow cytometry (B). The graphs represent data from one experiment with n=4 mice.*

Table 2: Effect of KRP203 in different allo-HSCT settings

≈ normal vs.CTRL and GVHD
↓: lower
↑: higher

CTRL = syn. Transfer (no GVHD)

CTRL =TCDBM (no GVHD)

BMT model			B6 → BDF1			Balb/c → B6		Balb/c → B6.RIPmOVA		129Sv → B6		B6 → Balb/c	
Conditioning			Non-irradiated			Irradiated		Irradiated		Irradiated		Irradiated	
Parameter analyzed			- GVHD	+ GVHD	+ GVHD + WD	- GVHD	+ GVHD	- GVHD	+ GVHD	- GVHD	+ GVHD	- GVHD	+ GVHD
		Origin	2 weeks	2 weeks	2 & 3 weeks	1.5-6 weeks	3-6 weeks	8 weeks	8 weeks	4 weeks	4 weeks	4 weeks	4 weeks
Thymic T-cell development	cell#	Donor BM-derived Host	normal	normal	normal	normal	≈ vs. CTRL ↑ vs. GVHD	normal	≈ vs. CTRL ↑ vs. GVHD	normal		normal	normal
	DN	Donor BM-derived Host	normal	normal	normal	normal	≈ vs. CTRL ↑ vs. GVHD	normal	≈ vs. CTRL ↑ vs. GVHD	normal	↑ vs. GVHD	normal	≈ vs.CTRL ↑ vs. GVHD
	DP	Donor BM-derived Host	↓ vs. CTRL	↓ vs. CTRL ↑ vs. GVHD	↓ vs. WT ↑ vs. GVHD	normal	≈ vs. CTRL ↑ vs. GVHD	normal	≈ vs. CTRL ↑ vs. GVHD	normal	↑ vs. GVHD	normal	≈ vs.CTRL ↑ vs. GVHD
	SP	Donor BM-derived Host	↑ vs. CTRL	↑vs. CTRL ↑vs. GVHD	↑vs. CTRL ↑vs. GVHD	normal	≈ vs. CTRL ↑ vs. GVHD	normal	≈ vs. CTRL ↑ vs. GVHD	normal	↑ vs. GVHD	normal	↓ vs. CTRL ≈ vs. GVHD
Donor T-cell infiltration	cell#	Donor		↑ vs. CTRL ↓ vs. GVHD	↑ vs. CTRL ↓ vs. GVHD		↑vs. CTRL ↓ vs. GVHD				↑ vs. CTRL ↓ vs. GVHD		≈ vs. GVHD
	frequency			↑ vs. CTRL ↓ vs. GVHD	↑ vs. CTRL ↓ vs. GVHD		↑vs. CTRL ↓ vs. GVHD				↑ vs. CTRL ↓ vs. GVHD		↑vs. CTRL ↑vs. GVHD
Peripheral T-cell development	spleen	Donor BM-derived Donor	normal	↑ vs. CTRL ↓ vs. GVHD	↑ vs. CTRL ↓ vs. GVHD	normal	**BM:** ↓ vs. CTRL ↑ vs. GVHD **Donor**: ↑ vs. CTRL ≈ vs. GVHD	normal	normal	normal	**BM:** ↓ vs. CTRL ↑ vs. GVHD **Donor**: ↑ vs. CTRL ≈ vs. GVHD	normal	**BM:** ↓ vs. CTRL ↑ vs. GVHD **Donor**: ↑ vs. CTRL ≈ vs. GVHD
	LN	Donor BM-derived Donor	normal	↑ vs. CTRL ↓ vs. GVHD	↑ vs. CTRL ↓ vs. GVHD	normal	**BM:** ↓ vs. CTRL ↑ vs. GVHD **Donor**: ↑ vs. CTRL ≈ vs. GVHD	normal	↑vs. CTRL ≈ vs. GVHD	normal	**BM:** ↓ vs. CTRL ↑ vs. GVHD **Donor**: ↑ vs. CTRL ≈ vs. GVHD	normal	**BM:** ↓ vs. CTRL ↑ vs. GVHD **Donor**: ↑ vs. CTRL ≈ vs. GVHD
TEC	cell#	Host	normal	↓ vs. CTRL ↑ vs. GVHD	↓ vs. CTRL ↑ vs. GVHD	normal	↓ vs. CTRL ↑ vs. GVHD	normal		normal	↓ vs. CTRL ↑ vs. GVHD	normal	↓ vs. CTRL ↑ vs. GVHD
	frequency		normal	normal	↓ vs. CTRL ↑ vs. GVHD	normal	↓ vs. CTRL ↑ vs. GVHD	normal		normal	↓ vs. CTRL ↑ vs. GVHD	normal	↓ vs. CTRL ↑ vs. GVHD
mTEC	cell#	Host	normal	↓ vs. CTRL ↑ vs. GVHD	↓ vs. CTRL ↑ vs. GVHD	normal	↓ vs. CTRL ↑ vs. GVHD	normal		normal	↓ vs. CTRL ↑ vs. GVHD	normal	↓ vs. CTRL ↑ vs. GVHD
	frequency		normal	normal	↓ vs. CTRL ↑ vs. GVHD	normal	normal	normal		normal	↓ vs. CTRL ↑ vs. GVHD	normal	↓ vs. CTRL ↑ vs. GVHD
Aire+mTEC	cell#	Host	normal	↓ vs. CTRL ↑ vs. GVHD	↓ vs. CTRL ↑ vs. GVHD	normal	↓ vs. CTRL ↑ vs. GVHD	normal		normal	↓ vs. CTRL ↑ vs. GVHD	normal	↓ vs. CTRL ↑ vs. GVHD
	frequency		normal	≈ vs. CTRL ↓ vs. GVHD	≈ vs.CTRL ↑ vs. GVHD	normal	↓ vs. CTRL ↑ vs. GVHD	normal		normal	normal	normal	↓ vs. CTRL ↑ vs. GVHD
GVL												↓ vs. GVHD ↓ vs. KRP	↑vs. CTRL ≈ vs. GVHD
autoreactive T cells	cell#								↑ vs. CTRL ↓ vs. GVHD				
	frequency								↑ vs. CTRL ↓ vs. GVHD				

**KRP203: Continuous and prophylactic administration (3mg/kg/2nd day)*