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Understanding and treatment of murine chronic Graft-versus-Host Disease

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Abbreviations

aGvHD: Acute Graft-versus-Host Disease
AIRE: Autoimmune Regulator
ALL: Acute Lymphocytic Leukemia
Allo-HSCT: Allogeneic Hematopoietic Stem Cell Transplantation
APC: Antigen-Presenting Cell
ATG: Anti-Thymocyte Globulin
BAFF: B Cell-Activating Factor
BCR-ABL: Breakpoint Cluster Region-Abelson Fusion Gene
CAR: Chimeric Antigen Receptor
CCL: Chemokine (C-C motif) Ligand
CFU: Clonogeneic Progenitor
cGvHD: Chronic Graft-versus-Host Disease
CIBMTR: Center for International Blood and Marrow Transplant Research
CML: Chronic Myeloid Leukemia
CNS: Central Nervous System
CR: Complete Response
CXCL: Chemokine (C-X-C motif) Ligand
CXCR: Chemokine (C-X-C Motif) Receptor
DC: Dendritic Cell
DLI: Donor Lymphocytes Infusion
DSHA: Donor Specific-HLA Antibodies
ERK: Extracellular-Signal-Regulated Kinase
FoxP3: Forkhead Box P3
G-CSF: Granulocytes Colony-Stimulating Factor
GvHD: Graft-versus-Host Disease
GvT effect: Graft-versus-Tumor Effect
HLA: Human Leukocyte Antigen
HSC: Hematopoietic Stem Cell
HSPCs: Hematopoietic Stem/Progenitor Cells
IFN-γ: Interferon-γ
IL-2Rγ: Interleukin-2 Receptor gamma
IL: Interleukin
LAP: Latency-Associated Protein
LTC-IC: Long-Term Culture-Initiating Cell
MHC: Major Histocompatibility Complex
miHA: Minor Antigen
MMF: Mycophenolate Mofetil
MR: Minor Response
MRD: Minimal Residual Disease
MSC: Mesenchymal Stem Cell
mTEC: Medullary Thymic Epithelial Cell

mTor: Mammalian Target of Rapamycin
MTX: Methotrexate
NC: Neurological Complications
NOD: Non-Obese Diabetic
NOD/SCID: Non-Obese Diabetic/Severe Combined Immune Deficiency
NRM: Non-Relapse Mortality
ORR: Overall Response Rate
ORR: Overall Response Rate
OS: Overall Survival
PAMPS: Pathogen-associated Molecular Patterns
PBSC: G-CSF-Mobilized Peripheral Blood Stem Cells
PDGF-r: Platelet-Derived Growth Factor-Receptor
PDGF: Platelet-Derived Growth Factor
PKC: Protein Kinase C
PR: Partial Response
RIC: Reduced Intensity Conditioning Regimen
ROS: Reactive Oxygen Species
RSF: Relapse-Free Survival
SCF: Stem Cell Factor
Scl-cGvHD: Sclerodermatous Chronic Graft-versus-Host Disease
Sle-cGvHD: Systemic Lupus-like Erythematous Chronic Graft-versus-Host Disease
SRC: SCID-Repopulating Cells
T conv: Conventional T cells
TCR: T-Cell Receptor
TFh: T Follicular Helper Cell
TGF-β-r: Transforming Growth Factor-β Receptor
TGF-β: Transforming Growth Factor-β
Th: T Helper
TKI: Tyrsosine Kinase Inhibitor
TNF-α: Tumor Necrosis Factor-α
TR1: Type 1 Regulatory T cells
TRA: Tissue-Restricted Peripheral Self-Antigen
T_{regs}: Regulatory T cells
UCB: Umbilical Cord Blood

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I. Introduction

I.1 Hematopoietic stem cells

I.1.1 Hematopoiesis

Hematopoiesis is a multi-step process by which blood cells (including red blood cells, neutrophils, lymphocytes, monocytes, platelets, …) are generated. All blood cells derive from a unique cell type: the hematopoietic stem cell (HSC). Bone marrow is the main site of hematopoiesis. However, the frequency of HSCs is low, ranging from 0.01 to 0.05 % of marrow cells. HSCs are able of self-renewal, maintaining throughout life a pool of HSCs. Further, when an increased demand occurs, HSCs are able to proliferate rapidly and under specific combinations of stimulating factors, HSCs can differentiate into a specific cell lineage [1]. Specifically, hematopoiesis is a four-step process. At the first stage of differentiation, HSCs become progenitors. After acquisition of specific characteristics, the progenitor becomes precursors, which are identifiable on a bone marrow sample. Finally, precursors progressively lose their proliferation capacity and reach maturity to become mature cells that are fully functional.

Two major types of progenitors exist: lymphoid and myeloid progenitors. Myeloid progenitors give rise to platelets, neutrophils, eosinophils, basophils, monocytes and red cells while lymphoid progenitors differentiate into B, T and NK cells (***Figure 1; p.11***).

After generation of immature lymphocytes in the bone marrow, lymphocytes will go through the maturation process. Maturation of B lymphocytes occurs in the bone marrow, while immature T cells go through this process in a distinct organ, the thymus. Following maturation, naive lymphocytes enter in the peripheral circulation and lymphoid organs for surveillance of invading pathogens and potential tumor cells.

B and T cells can differentiate upon exposure to an antigen/pathogen. Indeed, they can become effector and/or memory lymphocytes. Effector lymphocytes eliminate the antigen/pathogen (by antibody production, release of cytotoxic granules or modulation of the immune response) while memory cells remain in the periphery to facilitate the immune response to the same antigen upon future exposure [2].

I.1.2 Sources of HSCs for hematopoietic stem cell transplantation

HSCs can be obtained from three main sources: bone marrow, peripheral blood and umbilical cord blood.

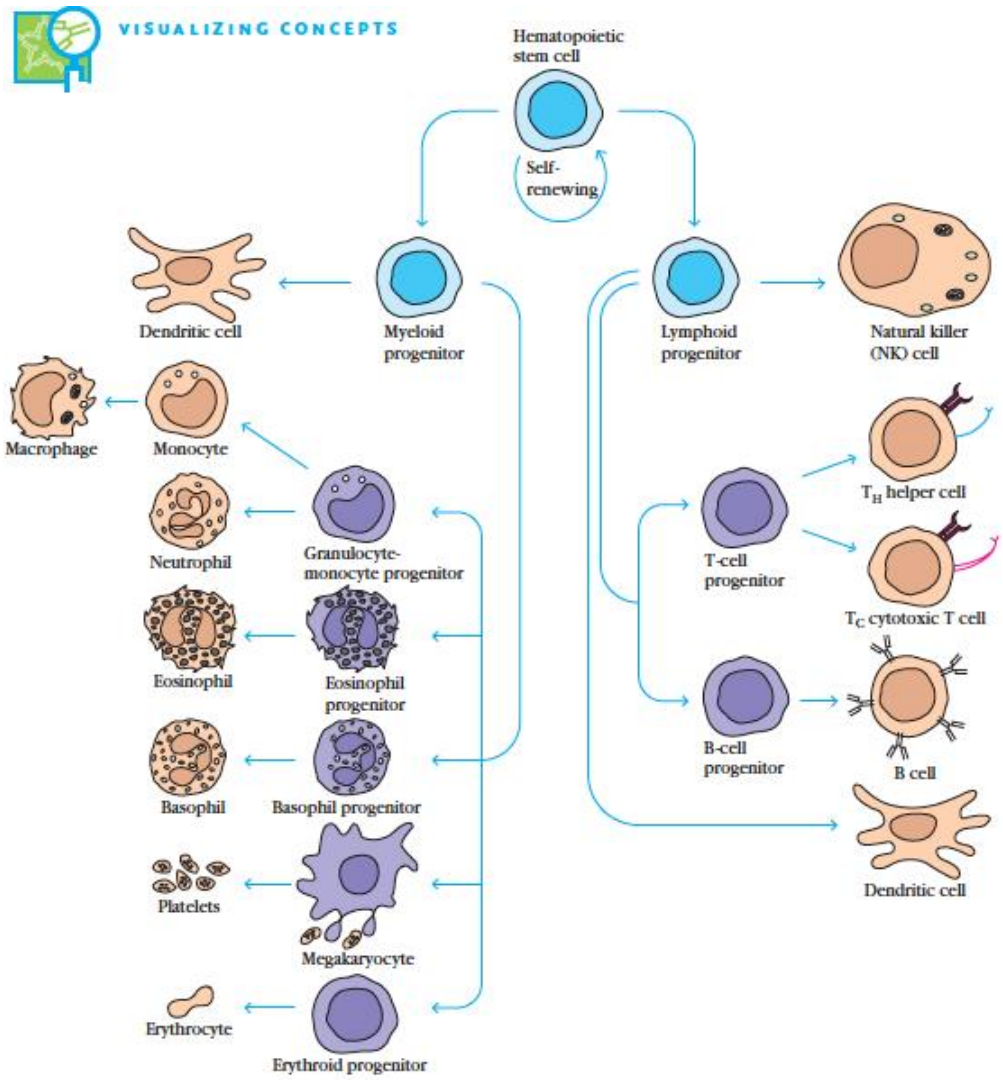

Figure 1: Hematopoiesis overview. From Immunology by *Goldsby [2]*

I.1.2.1 HSCs from bone marrow

Bone marrow is collected aseptically under general anesthesia. The aspirated bone marrow is aseptically filtered and then injected to the recipient with or without prior red blood cell depletion (depending on ABO compatibility between the donor and the recipient).

I.1.2.2 HSCs from peripheral blood

Donors first receive a treatment with granulocyte colony-stimulating factor (G-CSF) during 4-5 days. This treatment induces mobilization of HSCs to the blood (by depleting endosteal osteoblasts, leading to suppressed endosteal bone formation and decreased expression of factors required for HSC retention and self-renewal) [3]. Numbers of circulating HSCs will then increase in the periphery. After the 4-5 day treatment, mononuclear cells (including HSCs) are isolated from other blood cell types by cytapheresis. A new agent allowing HSC mobilization is currently increasingly used in the clinic: AMD3100. AMD3100 mobilizes HSCs by directly antagonizing the CXCR4 (Chemokine (C-X-C Motif) Receptor)-mediated sensing of the CXCL12 (Chemokine (C-X-C Motif) Ligand) chemotactic gradient in the bone marrow that maintains hematopoietic stem/progenitor cells (HSPCs) within this tissue, and by promoting the release of CXCL12 from bone marrow stromal cells into the circulation, thereby disrupting the CXCL12 gradient that retains HSCs within the bone marrow [3].

Peripheral blood HSCs (PBSCs) have some important advantages in comparison to bone marrow HSCs for both the donor and the recipient. Indeed, no general anesthesia of the donor is required, while engraftment and recovery of immunity are faster in PBSC recipients. However, PBSC recipients are also at higher risk of severe acute and chronic graft-versus-host disease (GvHD, discussed in next sections) [4, 5].

I.1.2.3 HSCs from umbilical cord blood

Umbilical cord blood (UCB) contains a high frequency of HSCs. However, absolute number of HSCs contained in a single UCB unit remains relatively low for transplantation in adults. This is the main reason why single UCB transplantation is often limited to small adults and children, when bigger adults can be offered double UCB transplantation [6].

UCB transplantation presents advantages and disadvantages. The main advantages of UCB transplantation include: low risk of infectious agent (such as cytomegalovirus) transmission, immediate availability reducing the delay for transplantation, tolerance of 1-2/6 human leukocyte antigen (HLA)-mismatch between the cord blood unit and the recipient allowing finding a donor and performing transplantation in patients from ethnic minorities, and a relatively low incidence of GvHD.

Disadvantages of UCB transplantation include slower engraftment (due mainly to the low absolute numbers of HSCs contained in a cord blood unit) [7, 8] and slower immune recovery [9] .

I.1.3 Mouse models for engraftment of human HSCs

Experimental identification of HSCs has been possible by *in vitro* culture of hematopoietic precursors during several weeks (called "long-term culture") [10]. This technique consists in the culture of HSCs with stromal cells, which reproduces *in vitro* the bone marrow microenvironment [11]. This assay allows the identification of very early progenitors, the LTC-IC (Long-Term Culture-initiating cell). These cells are more primitive than clonogeneic progenitors (CFU) produced in "short-term culture". CFUs and LTC-ICs are two distinct hematopoietic progenitors since they differ by their antigen expression at the cell surface. Further, growth factors needed for CFU or LTC-IC growth and maintenance are different, while proliferation rate is higher in CFUs than in LTC-ICs [12-17].

Repopulating activity of human HSCs have been extensively studied in irradiated (2-3 Gy) immunodeficient mice. NOD/SCID (Non Obese Diabetic/Severe Combined Immune Deficiency) mice lack functional B and T cells (*vide infra*). This lack of functional immune cells facilitates the colonization of the bone marrow microenvironment by human HSCs. Only primitive hematopoietic cells have the capacity to proliferate and generate both lymphoid and myeloid mature cells. These primitive transplanted cells are called SCID-repopulating cells (SRC). They represent < 0.1% of $CD34^+$ bone marrow cells [18]. However, although SRC are different from LTC-ICs, their exact place in the cellular hierarchy of hematopoiesis has remained unclear thus far [19]. Indeed, it was demonstrated that the frequency of SRC among bone marrow nucleated cells is 100 times lower than that of LTC-ICs [20]. Furthermore, while *in vitro* culture of LTC-ICs allowed a significant expansion, SRCs expansion has met with little success thus far [21, 22].

The $Prkdc^{scid}$ gene was one of the first mutations resulting in severe combined immunodeficiency in a CB17 mouse. This mutation results in a loss-of-function mutation of the Prkdc gene coding for the catalytic subunit of a DNA-dependent protein kinase. This protein kinase has a role in resolving the DNA double strand breaks that occur during V(D)J recombination. The resulting phenotype of the transgenic mice is unable to produce functional surface receptors on mature T and B cells [23, 24]. However, only low levels of engraftment are achieved after transplantation of human cells in these mice [25-27].

Non-obese diabetic (NOD) mice with *scid* mutation ($Prkdc^{scid}$) showed an altered antigen expression, imperfect myeloid lineage production, lack of complement and low NK cell activity, with better engraftment levels of human cells [28-30]. However, the NOD/SCID mouse model is imperfect. Indeed, immunodeficiency in NOD/SCID mice is incomplete since functional NK cells persist in the blood. Moreover, lack of T and B cells limit their use and around 70% of these mice develop thymic lymphomas, severely decreasing their life span [29, 31]. To

circumvent this NK cell activity, NOD/SCID mice were backcrossed with β2-microglobulin-deficient mice resulting in NOD/SCID-B2m$^{-/-}$ mice. The B2m$^{-/-}$ allele results in the absence of MHC class I expression and thereby loss of NK cell activity and better engraftment of human cells. Unfortunately, the NOD/SCID-B2m$^{-/-}$ mice develop lymphomas even faster than NOS/SCID mice and hemochromatosis [32, 33].

Interleukin-2 receptor gamma (IL-2Rγ) is responsible for signal transmission within the αβγ-complex of interleukins. A complete *null* mutation of this IL-2Rγ eliminates the signaling through the γ-chain and thus impairs NK- (and T-) cell development [34, 35]. Consequently, NOD/SCID-*IL-2Rγ*$^{-/-}$ mice were shown to allow the higher percentage of human engraftment, while, importantly, they are much less prone to thymic lymphomas [34, 36].

I.2 Allogeneic hematopoietic stem cell transplantation

I.2.1 Definition

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) remains the best treatment option for several hematological malignancies and some genetic diseases affecting blood cells. Allo-HSCT consists in a conditioning regimen (irradiation and/or chemotherapy) followed by transplantation of a graft composed of HSCs, hematopoietic progenitors, and mature immune cells such as lymphocytes and dendritic cells [37].

Several types of allo-HSCT exist depending on the stem cell source (bone marrow, mobilized peripheral blood or umbilical cord blood) and HLA compatibility between donor and recipient (HLA-matched or mismatched). The choice of the type of allo-HSCT depends on several criteria including recipient's disease, age, and available donor/graft [37].

I.2.2 Complications of allo-HSCT

Patients receiving allo-HSCT are submitted to several potential complications including graft rejection, toxicities of the conditioning regimen, infections, and GvHD. GvHD will be discussed later in this introduction, while toxicities of the conditioning regimen and infections are outside the topic of this thesis.

Immune graft rejection is caused by recipient immune cells recognizing donor minor and/or major histocompatibility antigens. B, NK and T cells are all implicated in graft rejection following HLA-mismatched allo-HSCT, while only T cells seem to be implicated following HLA-matched HSCT. Two types of rejection can occur in the context of HLA-mismatched allo-HSCT. First, an early (first days after

transplantation) rejection, mediated either by recipient NK cells (that are relatively radio-resistant) or by pre-existing donor-specific anti-HLA antibodies. Secondly, a late rejection, mediated by donor T cells [38, 39].

The main risk factors for graft rejection include a high number of prior transfusions, HLA disparities between donor and recipient (and particularly HLA class 1 disparities), use of an unrelated donor, T-cell depletion from the graft, low doses of transplanted hematopoietic stem cells, low doses of transplanted immune cells, low intensity of the conditioning regimen (such as Reduced Intensity Conditiong Regimen (RIC)), and presence of donor-specific anti-HLA alloantibodies. There is still some controversy as to whether alloantibodies actually mediate graft rejection, or if they are surrogate markers for cellular immunity that cause graft failure. However, it has been demonstrated that anti-HLA antibodies cause graft failure in animal models of allo-HSCT mainly because the cognate HLA antigens are expressed on HSCs and hematopoietic precursors. Clinical studies have also demonstrated that the presence of anti-HLA antibodies was significantly associated with graft rejection, indicating that the presence of pre-transplantation donor HLA-specific antibodies (DSHAs) in recipients of unrelated donor HSCT should be considered in donor selection [39, 40].

I.2.3 The Graft-versus-Tumor effect

The success of allo-HSCT is mainly based on the Graft-versus-Tumor effect (GvT effect). However, the biology of this effect remains partially unsolved. GvT effects are mainly attributed to donor T cells. However, donor NK cells might also play an important role, particularly in the context of T cell-depleted allo-HSCT and HLA-mismatched transplantation [41].

Evidence that GvT effects are primarily mediated by donor T cells came from the observation that the incidence of relapse was dramatically increased by T-cell depletion of the graft. Further, the ability of Donor Lymphocyte Infusions (DLI) to induce remissions in patients who relapsed after allo-HSCT definitively provided direct evidence that the GvT effect is mediated by mature donor immune cells [42, 43]. However, complex interactions with several immune cell types, antibodies and cytokines are also important. It is also important to note that GvHD and GvT effects are closely linked since the same donor cell populations are responsible for these two immunological effects, and occurrence of GvHD has been associated with lower risks of relapse [44, 45]. However, the separation of beneficial GvT effects and deleterious GvHD has remained a major challenge for researchers [46].

The nature of recognized antigens remains a focus of intense research. The fact that GvHD and GvT effects are closely linked suggests that the main mechanisms of GvT effects are the recognition of host-specific minor or major histocompatibility

antigens by donor T cells. Indeed, the risk of relapse is significantly higher after syngeneic bone marrow transplantation both in humans and in the mice. In that setting, cells contained in the graft are genetically similar to recipient malignant cells. As the donor graft contains mature cells, they have received thymic education preventing them to react against "the self" (tolerance). As these donor mature cells are unable to eliminate recipient malignant cells, the relapse rate is higher. However, several tumor-associated antigens (such as p210 BCR-Abl, WT1 [47-49]) or minor-histocompatibility antigens (miHAs) with restricted expression to hematopoietic tissues (such as HA-2, HB-1, LRH-1 [50-52]) have also been identified, opening the way to donor vaccination strategies aimed at inducing specific GvT responses [46] or to strategies of donor T-cell modifications.

CAR (Chimeric Antigen Receptor)-modified T cells are generated through gene modification, using retroviral vectors, of T cells to confer specificity against certain antigens. This approach has been successful for targeting T cells to lymphoma, leukemia, melanoma, viruses, miHAs, and oncoproteins. Such engineered T cells acquire the antigen specificity of the introduced T-cell Receptors (TCRs), including the ability to lyse antigen-bearing target cells and to eliminate tumors *in vivo*. The preclinical and initial clinical studies of TCR gene transfer have shown promise but have also revealed limitations. Indeed, sufficient levels of expression of the transferred TCR α and β chains are not always accomplished with currently available vectors. The introduced TCR chains can also cross-pair with the endogenous TCR chains, resulting in formation of hybrid receptors with unknown and potentially deleterious specificity. Fortunately, this problem may be resolved using murine rather than human constant regions in the introduced TCR chains, or by incorporating cysteine residues in the human constant regions of both the α and β chains. However, the avidity of the receptors may still be low if the TCR genes are cloned from a T-cell specific for a tumor-associated self-antigen [53-55].

Finally, the discovery of the importance of GvT effects has prompted the development of new approaches for allo-HSCT based on the administration of a RIC regimen. After RIC allo-HSCT, tumor eradication relies mainly on the GvT effect. However, GvT activity may also vary in intensity depending on the immunogenicity of the tumors and the respective proliferation rates of tumor cells and donor immune cells [56-58]. These approaches allowed performing allo-HSCT in older or weaker patients, who cannot support classical myeloablative conditioning regimens. RIC allo-HSCT is associated with lower non-relapse mortality than classical allo-HSCT, but also with a higher risk of relapse. Based on the GvT effect, the use of pre-emptive DLI after HSCT also allows under some circumstances to prevent or treat post-transplant relapses of the primary diseases occurring after allo-HSCT [59-61].

I.3 Graft-versus-Host Disease

Unfortunately, GvHD has remained a major complication of allo-HSCT [62]. Specifically, GvHD has a mortality rate of 15-30% and remains the main cause of morbidity after allo-HSCT.

In 1966, Billingham published three prerequisites for GvHD development: (1) the graft must contain immunocompetent cells, (2) the recipient must express tissue antigens that are not present in the donor, and (3) the patient must be incapable to mount an effective immunological response against the transplanted cells [63]. GvHD can thus be considered as an exaggerated manifestation of normal inflammatory mechanisms in which donor T lymphocytes respond to antigens that are specific to host cells. These antigens can be major histocompatibility complex (MHC) antigens (in case of MHC mismatch(es) between the donor and the recipient), or minor histocompatibility antigens (miHAs, see below). Indeed, in humans, MHC proteins are particularly highly polymorphic [62, 64, 65].

Two types of GvHD have been described: acute GvHD (aGvHD), which occurs classically within 100 days after transplantation, and chronic GvHD (cGvHD), that typically develops after D+100 post-transplantation. The next paragraphs will describe the pathogenesis, clinical picture, and treatment of acute and chronic GvHD.

I.3.1 Acute Graft-versus-Host Disease

I.3.1.1 Clinical features

In a large registry study from the Center for International Blood and Marrow Transplant Research (CIBMTR), the incidences of grade II-IV and grade III-IV acute GvHD were 34% and 16%, respectively, in sibling recipients, and 52% and 21%, respectively, in HLA-matched unrelated recipients [66].

Risk factors for aGvHD include donor/recipient HLA incompatibility, higher donor/recipient age, female donor to male recipient, unrelated donor and PBSC as HSC source (for grade III-IV aGvHD) [67]. In contrast, the incidence and severity of aGvHD are attenuated with the use of UCB as stem cell source compared to the use of bone marrow [68, 69].

The diagnosis of aGvHD was until recently arbitrarily defined based on the timeframe post-transplantation. Specifically, aGvHD was historically defined as GvHD occurring before 100 days after transplantation [70]. Nowadays, aGvHD is defined as GvHD with inflammatory processes involving the skin, liver, and/or the gastro-intestinal tract, irrespectively of the timing of occurrence but without signs of cGvHD. Acute GvHD is divided into four grades, mild (grade I), moderate (II),

severe (III) and life-threatening (IV) [71]. Four-year overall survivals are 80%, 25% and 5% for grades, II, III and IV, respectively [72].

The skin is the most commonly affected organ. Skin aGvHD is characterized by an erythematous maculopapular rash. In the most severe cases, the skin blisters and ulcerates. Histopathology demonstrates apoptosis at the basis of epidermal rete ridges, dyskeratosis, lymphocyte exocytosis, and perivascular lymphocytic infiltration in the dermis [70, 73-75]. Acute GvHD in the gastro-intestinal tract symptoms causes nausea, vomiting, anorexia, diarrhea and/or abdominal pain. Histology reveals patchy ulcerations, apoptotic bodies at the base of crypts, abscesses in these crypts and loss of the epithelium surface [76]. Finally, liver aGvHD is a very challenging diagnosis since it shares clinical features with other causes of liver dysfunction such as drug toxicity, iron overload, or veno-occlusive disease of the liver. Histologic features of liver aGvHD include endothelialitis, pericholangitis, bile duct destruction and lymphocytic infiltration [77, 78].

I.3.1.2 Prophylaxis

Prophylaxis of GvHD is generally based on calcineurin inhibitors, such as cyclosporine and tacrolimus, usually combined with an antimetabolite (methotrexate (MTX) or mycophenolate mofetil (MMF)). Described in 1986, the cyclosporine plus MTX combination has demonstrated its superiority in reducing aGvHD incidence and increasing overall survival (OS) compared with either agent alone [79-83]. Cyclosporine and tacrolimus exert their function by inhibiting the calcineurin c-enzyme, essential for T-cell activation. However, these molecules induce a number of adverse effects such as hypomagnesemia, hypertension, tremor, and last but not least, nephrotoxicity [84].

Several studies investigated new drugs for GvHD prophylaxis. Among these, Sirolimus (also called rapamycin) is a mammalian Target of Rapamycin (mTor) inhibitor commonly used as an immunosuppressant in solid organ transplantation. The rationale for sirolimus in GvHD prophylaxis is based on its different mechanisms of action resulting in non-overlapping side effects with calcineurin inhibitors, and its ability to promote regulatory T cells (T_{regs}) [85, 86]. Sirolimus and its mechanisms of action will be discussed later in more details.

Ex vivo T-cell depletion (based on negative selection techniques or on positive selection of $CD34^+$ cells) of the graft remains the best method for preventing GvHD. However, this strategy increases the risk of graft rejection, relapse, life-threatening infection, and EBV-induced lymphoproliferative disorders [87-89]. *In vivo* T-cell depletion can also be performed by adding antithymocyte globulin (ATG) to the standard prophylaxis regimens [90], apparently preventing severe GvHD without increasing the risk of relapse when appropriate doses are administered [91, 92]. Alemtuzumab (anti-CD52 IgG1 monoclonal antibody; CD52 is present on mature

lymphocytes and monocytes but not on HSCs) in combination with cyclosporine has also been very successful at preventing both aGvHD and cGvHD but increased the risk of infection and relapse [93, 94], at least when high doses were used. However, as observed with ATG [91, 92], recent studies suggest that moderate doses of alemtuzumab might allow preventing severe GvHD without increasing the relapse risk in the setting of allogeneic PBSC transplantation [91].

Azacitidine, post-transplantation cyclophosphamide, anti-TNF agents, and bortezomib are currently investigated for their potential role in GvHD prevention [95]. Azacitidine is a hypomethylating agent that increased T_{reg} numbers by demethylating the FoxP3 gene and by slightly inhibiting T-cell proliferation [96]. Initially tested after haplo-identical HSCT, early post-transplantation cyclophosphamide administration (a highly cytotoxic molecule for proliferating T cells but not for resting T lymphocytes nor for T_{regs} [97]) in HLA-matched bone marrow transplantation allowed a significant reduction in both grade III-IV aGvHD and cGvHD [98]. However, as T cells involved in the GvT effect are also activated, cyclophosphamide could also kill these beneficial cells. Tumor Necrosis Factor- α (TNF-α) inhibitors (infliximab, etanercept) might also be useful since increase in TNF-α receptor expression is observed early in the development of GvHD. However, prophylaxis with infliximab or etanercept in combination with standard prophylaxis failed to reduce aGvHD incidence [99, 100]. Finally, selective depletion of alloreactive T cells without inhibitory effects on T_{regs} could be also achieved with bortezomib. A lower incidence of GvHD was observed when patients received standard prophylaxis in combination with bortezomib [101].

Cellular approaches such as mesenchymal stem sell (MSCs) or T_{reg} infusions are also investigated as methods of GvHD prophylaxis. Both cell types are immunoregulatory cells that inhibit T-cell proliferation and dendritic cell differentiation. MSCs are multipotent progenitors found within the bone marrow, adipose tissue, fetal membranes and umbilical cord. They seem to exert their immunoregulatory properties through the release of immunomodulatory molecules such as prostaglandin E2, Interleukin (IL)-10, Transforming Growth Factor-β (TGF-β), nitric oxide, and by promoting T_{regs} [102, 103]. Several studies in GvHD murine models, including xenogeneic GvHD models, have produced conflicting results (review in [104]). Further, small pilot clinical studies have observed encouraging results that need to be confirmed in appropriate double blind randomized studies. The potential impact of T_{reg} infusion on GvHD prevention/treatment will be discussed in a separate section (vide infra).

I.3.1.3 Pathophysiology

The pathogenesis of aGvHD pathogenesis is now better understood (at least in mice). It has been shown that donor T cells mediate aGvHD while some recipient cells (such as B cells and dendritic cells) and cytokines also play a role by activating

donor conventional T cells (T conv). Four different steps have been suggested: (1) activation of Antigen-Presenting Cells (APCs); (2) donor T-cell activation, proliferation, differentiation and migration, (3) target tissue destruction, and (4) escalation of disease severity by a spiraling process [105]. These different phases are briefly described below (***Figure 2***).

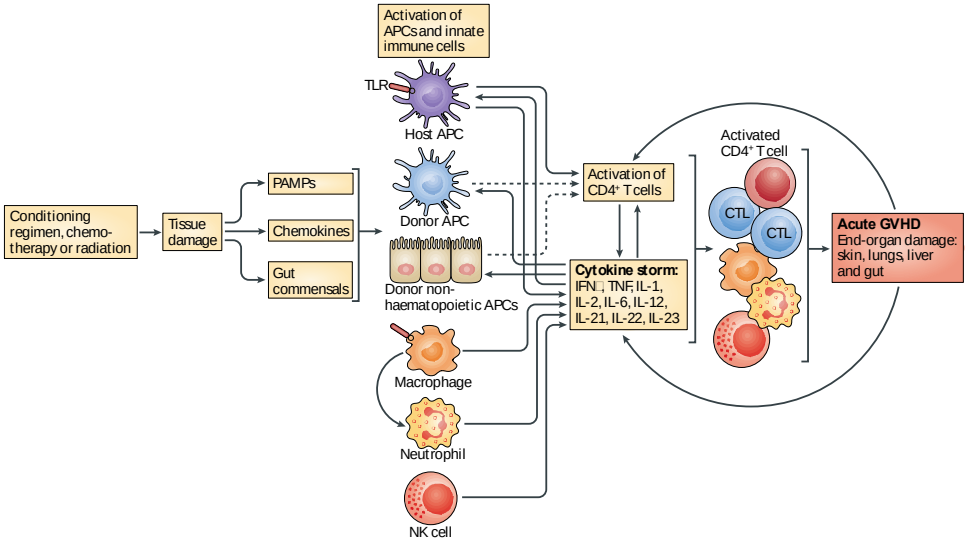

Figure 2: Acute GvHD pathogenesis (from Blazar et al., 2012). Initiation and maintenance of aGvHD have been conceptualized into 4 phases with feedback loops that self-sustain the process. Although the effect of the conditioning phase in aGvHD is not absolutely necessary, in many of the models it activates APCs, via tissue destruction, and improves APC capacity. It can also, through release of gut bacteria, Pathogen-Associated Molecular Patterns (PAMPS) and chemokines, activate cellular components of the innate immune system that can participate in direct tissue damage and contribute to the cytokine storm. Host hematopoietic APCs have perhaps the most important role in initiation of GvHD, but this may depend on the model and the potential role of recipient APCs as well as host non-hematopoietic APCs should not be ignored. Following antigen presentation, a strong cytokine response is initiated, promoting greater antigen presentation and recruitment of effector T cells and innate immune cells further contributing to the inflammatory cytokine milieu. Finally, effector T cells, NK cells, macrophages and pro-inflammatory cytokines (e.g. TNF- α), will result in end-organ damage, clinically recognized as aGvHD in the skin, lung, gut and liver. The resulting tissue damage, if not treated, will further escalate the disease, spiraling up the process to higher and more severe stages of GvHD pathology, which is extremely difficult to control.

I.3.1.3.1 Phase 1: activation of APCs

Conditioning regimens contribute to primary tissue injury resulting in subsequent proinflammatory factor release, such as TNF-α, IL-1, CCL2 (chemokine (C-C) motif ligand), CCL3, CCL4, CCL5, CXCL9, CXCL10 and CXCL11, leading to an increased expression of adhesion molecules, MHC antigens and costimulatory molecules on APCs [106].

I.3.1.3.2 Phase 2: donor T-cell activation

Donor T cells are activated upon recognition of alloantigens presented by primed host/donor APCs. These interactions occur mainly in secondary lymphoid organs [107, 108]. Signaling through the TCR induces a conformational change in adhesion molecules, resulting in solid interactions between T-cell costimulatory molecules and their ligands on APCs facilitating T-cell activation [109, 110]. Activated T cells then secrete Th1/2/17 cytokines. Th1/Th2 cytokine balance is important for the pathogenesis of GvHD. Indeed, Th1 cytokines are efficient inducers of aGvHD. For example, TNF-α and IL-2 could amplify T-cell activation and proliferation [73, 82, 111]. Th2 cytokines could inhibit aGvHD development. Indeed, data suggest that increased IL-10 production by recipient cells stimulated *ex vivo* could decrease the risk of aGvHD [112]. In conclusion, multiple cytokines play a role in the regulation and development of aGvHD. However, the timing and the duration of the expression of these cytokines seem to be critical [105].

T_{regs} play also an important role. Indeed, murine aGvHD models and clinical studies have demonstrated that addition of donor T_{regs} to the graft prevented aGvHD [113-115].

I.3.1.3.3 Phase 3: cellular and inflammatory effector phase

This phase implicates a large variety of mediators responsible for the amplification of target tissue damages. Both innate and adaptive immune systems are implicated during this phase. However, as mentioned above, cytotoxic T cells are the main cellular effector of aGvHD by lysing target cells. They use principally the granzyme/perforin and the Fas/FasL systems [116].

During phases 1 and 2, increased expression of costimulatory signals induce production of chemokines. These signaling molecules will induce migration of donor T cells from lymphoid tissues to the target organs [62, 117]. Chemokine gradient and adhesion molecules are important during this process. Implicated chemokines include CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10 and CXCL11. Involved adhesion molecules include mainly L-selectin and $\alpha_L\beta_2$ integrin.

Acute GvHD injury could also result from the secretion of inflammatory cytokines, such as TNF-α and/or IL-1. TNF-α is produced by both donor and recipient cells and has several key roles: (1) activation of APCs resulting in increased alloantigen presentation, (2) recruitment of effector cells in target organs and (3) induction of tissue injury through apoptosis and necrosis of host cells [118]. IL-1 secretion occurs mainly in the spleen and skin and has also been associated with the development of aGvHD [119].

I.3.1.3.4 Phase 4: Feedback loop

Effector cells, including T cells, NK cells, macrophages, and pro-inflammatory cytokines will damage target organs like the skin, lung, gut and liver. If not treated, these damages will sustain the disease, inducing more severe stages of GvHD pathology [120].

I.3.1.4 Treatment

Acute GvHD mortality depends mainly on the severity of the disease (stage IV > stage III > stage II), and the response of aGvHD to first-line treatment. Usually first-line treatment consists of high-dose corticosteroids (prednisolone or methylprednisolone 1-2 mg/kg for 7 days with tapering according to GvHD response). Around 25 to 40% of patients completely respond to this treatment.

Unfortunately, for the 60-75% remaining non-responding patients, no standard second-line treatment exists, although several second-line treatments have been assessed in small phase II studies and induce GvHD responses (defined by 6-month survival from initiation) in half of the patients [95]. These treatments include immunosuppressive drugs (shift from cyclosporine to tacrolimus, sirolimus, cyclophosphamide and mycophenolate mofetil), extracorporeal photopheresis, monoclonal antibodies against IL-2 (basiliximab, daclizumab, inolimomab or denileukin) or against TNF-α (etanercept), alemtuzumab and ATG. Interestingly, a recent meta-analysis failed to demonstrate that any of these treatments were better or worse than another [121].

Infusion of MSCs or of *in vitro* expanded T_{regs} is also currently investigated as a treatment for aGvHD [104, 122]. The safety and efficacy of MSC infusion in steroid-refractory aGvHD patients has been suggested in a nice clinical study from the European Group for Blood and Marrow Transplantation (EBMT). Around 54% and 16% of patients achieved complete (CR) or partial responses (PR) without significant side effects. Median time for CR was 18 days post MSC infusion [122].

I.3.2 Chronic Graft-versus-Host Disease

I.3.2.1 Clinical features

Chronic Graft-versus-Host Disease is the main cause of late death and morbidity after allo-HSCT and may impair severely quality of life [123, 124]. Incidence ranges from 30-45% in transplants with a HLA-identical sibling donor to 70-80% with an unrelated mismatched donor. In a large CIBMTR study, cGvHD was observed in 42% of the sibling recipients and in 49% of HLA-matched unrelated recipients [66]. Besides HLA-mismatching, risk factors for cGvHD include prior aGvHD, high recipient age, female donor to male recipient, and use of PBSCs [123, 125].

cGvHD presentation can be progressive (developing following unsolved aGvHD), quiescent (occurring following cured aGvHD) or *de novo*. Historically, the diagnosis of cGvHD (rather than aGvHD) was based on the timing of symptom onset (> 100 days post-transplantation) and the disease was divided into a "limited" (localized skin without skin sclerosis and/or mild liver involvement) or an "extensive" form [126]. This classification was not optimal and, recently, the National Institutes of Health (NIH) Consensus Development Project established in 2005 new criteria based on signs and not on timing [127]. These criteria were recently updated [128].

In the 2005 NIH classification, cGvHD diagnosis is based on the presence of at least one diagnostic clinical sign of cGvHD or the presence of at least one distinctive clinical manifestation confirmed by biopsy or other relevant tests in the same or another organ (***Table 1; p.24***) [73, 127]. Further, cGvHD can be divided into classical cGvHD (without any sign of aGvHD) or overlap syndrome (where signs of aGvHD and cGvHD coexist). GvHD severity is based on severity in each affected organ and cGvHD is divided into mild, moderate or severe.

cGvHD share symptoms with autoimmune diseases such as scleroderma, systemic lupus erythematosus, Sjögren syndrome, and/or can affect the lung where it causes a syndrome similar to what is observed in lung transplant rejection, termed bronchiolitis obliterans. Skin is the most frequently affected organ at the time of diagnosis [129]. Skin lesions are characterized by a mononuclear infiltrate leading to destructive changes at the dermal-epidermal junction progressing into fibrosis and sclerosis [130].

Prevention of cGvHD can be achieved, among others, by using ATG, alemtuzumab, *in vitro* T-cell depletion of the graft, or post-transplantation cyclophosphamide (described above in the aGvHD section). Moreover, several first-line and second-line treatments exist. Treatment of cGvHD can be systemic or topical. Systemic treatment is generally given for moderate or severe cGvHD, while topical treatment is generally given alone in mild cGvHD and is often combined with systemic

treatment in moderate/severe cGvHD. The most-widely used first-line therapy for moderate/severe cGvHD is a combination of corticosteroids (the only first-line medication that has been demonstrated to prolong survival of cGvHD patients) and calcineurin inhibitors (tacrolimus, cyclosporine). Unfortunately, only 20 to 50% of patients achieved complete withdrawal of systemic treatment. Moreover, steroids often induce important sides effects (diabetes, myopathy, avascular necrosis, osteoporosis, weight gain with changes in body habitus, cataract and emotional lability) and cause severe immunodeficiency that can lead to fatal infectious complications [131-133].

Skin	Dyspigmentation, new onset alopecia, poikiloderma, lichen planus-like eruptions or sclerotic features
Nails	Nail dystrophy or loss
Mouth	Xerostomia, ulcers, lichen-type features, restrictions of mouth opening from sclerosis
Eyes	Dry eyes, sicca syndrome, cicatricial conjunctivitis
Muscles, fascia, joints	Fasciitis, myositis, or joint stiffness from contractures
Female Genitalia	Vaginal sclerosis, ulcerations
GI tract	Anorexia, weight loss, esophageal web or strictures
Liver	Jaundice, transaminitis
Lungs	Restrictive or obstructive defects on pulmonary function tests, bronchiolitis obliterans, pleural effusions
Kidneys	Nephrotic syndrome (rare)
Heart	Pericarditis
Marrow	Thrombocytopenia, anemia, neutropenia

Table 1. Signs and Symptoms of Chronic GvHD (from reference 73)

Unfortunately, there is no standard second-line treatment for patients with steroid-refractory cGvHD, although several second-line treatments have been investigated in phase II studies. Typically, these phase II studies included few patients with heterogeneous organ involvement, and in advanced phases [134-141]. Second-line treatments include immunosuppressors (MMF, MTX, alemtuzumab), immunomodulating approaches (photophoresis, mTOR inhibition (sirolimus or everolimus), thalidomide), thoraco-abdominal irradiation, antifibrotic agents (imatinib, nilotinib) and rituximab. New therapeutic approaches such as low dose IL-2 injection, MSC and T_{reg} infusions are also currently tested.

Unfortunately, none of the second-line treatment has proven yet to be more efficient than another (or than placebo) in appropriate randomized trial(s). Consequently, as no predictor factor for efficacy of a specific agent exists, the “trial-and-error” approach has remained the usual way to identify the drug effective in the individual patient [133].

I.3.2.2 Pathophysiology

Chronic GvHD is a multisystemic disorder in which a myriad of manifestations occurs in several organs [142]. Although the pathophysiology of cGvHD remains not fully understood, recent studies have identified six pivotal actors in its development: damages to the thymus impairing T-cell selection, Th2 cells and cytokines, macrophage activation, T_{regs}, B cells and fibroblasts (***Figure 3***).

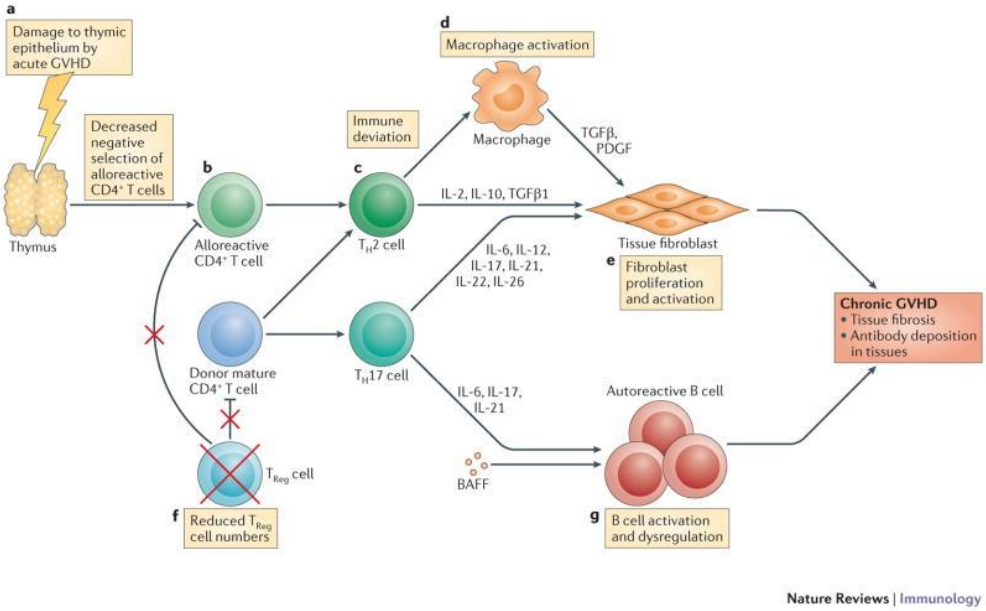

Figure 3: cGvHD pathogenesis (from Blazar et al., 2012). The pathophysiology of cGvHD (at least in some murine models) mostly revolves on the polarization of T helper cells to a Th2 cytokine phenotype but there are six hallmarks that are unique to this syndrome. These include damage to the thymus associated with the conditioning regimen and more importantly, occurrence of aGvHD earlier in the post-HSCT course resulting in decreased negative selection of alloreactive $CD4^+$ T cells; Th2 cytokine pattern deviation resulting in release of fibrogenic cytokines such as IL-2, IL-10 and TGF-β; macrophage activation followed by tissue fibroblast proliferations and activation through release of TGF-β and Platelet-Deerived Growth Factor (PDGF) from macrophages; lower T_{reg} levels; and finally, dysregulation of B cells leading to emergence of autoreactive B cells and production of autoreactive antibodies. It suggested that the latter may be due to excessive presence of B-cell Activating Factor (BAFF) in the lymphoid microenvironment. All this will results in an autoimmune-like systemic syndrome mostly associated with fibroproliferative changes that can occur in almost any organ but primarily affects oral and ocular mucosal surfaces and the skin, lung, liver and gut. Recent observations have also unraveled a major role for Th1 and Th17 cells, as well as for T follicular helper cells (Tfh) [143].

I.3.2.2.1 Conventional T cells

Donor T cells that recognize genetic disparities between the donor and recipient or that recognize self-antigens are the primary mediators of cGvHD [144, 145].

T-cell reconstitution after transplantation: impact of the thymus on cGvHD

T-cell repopulation following HSCT results from both thymic-dependent and thymic-independent pathways. It is now obvious that the thymic-independent peripheral expansion of mature T cells is responsible for the development of both aGvHD and cGvHD because T-cell depletion of the graft reduces rates of both aGvHD and cGvHD [146]. However, it has been postulated that donor-derived T cells generated from hematopoietic stem cells via the recipient's thymus could also play a role in cGvHD pathogenesis.

Within the thymus, T cells undergo both positive and negative selection, thus resulting in the elimination of self-reactive cells. While positive selection is mediated by the thymic cortical epithelium, negative selection is mediated by thymic dendritic cells (DCs) to eliminate self-reactive T cells via clonal deletion [147, 148]. The exposure of thymocytes to self-antigens (including those with highly restricted tissue expression) is essential for clonal deletion. Within the thymus, many tissue-restricted peripheral self-antigens are expressed by mature medullary thymic epithelial cells ($mTEC^{high}$). Tissue-restricted peripheral self-antigen (TRA) expression is controlled partly by the transcription factor autoimmune regulator (AIRE). It was demonstrated that both deficits in AIRE and/or TRA expression impair negative selection and can consequently cause autoimmune disease.

It has long been hypothesized that defects in thymic negative selection might have a role in cGvHD development. Studies have demonstrated that the thymus could be a target of aGvHD [149]. Interestingly, the development of human aGvHD predisposes to cGvHD. Experimental data indicate that thymic damage by cGvHD results in a loss of thymic negative selection [150]. Indeed, a hallmark of murine aGvHD is the *de novo* generation of autoreactive T cells from donor HSCs, which can mediate the evolution from acute to cGvHD. Because the thymic epithelium is a target of donor T-cell alloimmunity, *Dertschnig et al*. have demonstrated that thymic aGvHD interferes with the $mTEC^{high}$ capacity to sustain TRA diversity [151]. In this study, both a marked reduction of $AIRE^{+}$ mTEC and a decrease in the diversity of AIRE-dependent TRA were observed. Moreover, 2 other studies confirmed that impaired thymic negative selection due to aGvHD could lead to the emergence of an autoreative $CD4^{+}$ T-cell subpopulation causing cGvHD [150, 152]. Interestingly, in these models thymectomy prevented cGvHD [152]. Finally, evidence that GvHD could cause thymic damage includes the observation of a lower thymic activity (assessed by sjTREC levels) in patients with current or prior acute or chronic GvHD [149, 153-156].

In addition, thymectomy of 3-day-old mice results in clinical manifestations similar to cGvHD [157, 158]. Moreover, destruction of normal thymic architecture by aGvHD could also impair T_{reg} development [159-162]. Indeed, it was found that Hassall's corpuscles (or thymic corpuscles (bodies)) are crucial sites for T_{reg} development [157, 163].

Impact of Th subsets

Th1 and Th2 $CD4^+$ T-cell subsets can be identified by the cytokine pattern they produce. Interferon-ɣ (IFN-ɣ) is the hallmark of Th1 T cells while Th2 T-cell subpopulations produce IL-4, IL-5, Il-10 and Il-13 [164]. In contrast to aGvHD, the cytokine pattern observed in cGvHD is mainly Th2 [165]. Th2 cytokine expression results in release of fibrogenic cytokines such as IL-4, IL-10 and TGF-β [120]. These cytokines will in turn induce collagen synthesis by fibroblasts, as well as fibroblast activation and proliferation.

Recently, *Nishimori et al.* also observed upregulation of Th17 cells in a MHC-compatible, miHA-incompatible murine model of cGvHD. Further, transplantation of IL-$17^{-/-}$ cells in the same murine model was associated with milder skin and salivary gland cGvHD than following transplantation of wild-type cells, suggesting that Th17 T cells may play a role in cGvHD pathogenesis [166].

T follicular helper cells

Tfhs are antigen-experienced $CD4^+$ T cells found within B-cell follicles of secondary lymphoid organs. They are identified by their constitutive expression of CXCR5 (B-cell follicle homing receptor). Upon cellular interaction with B cells, Tfhs trigger the formation and maintenance of germinal centers through the expression of CD40L, IL-21 and IL-4. Tfhs are crucial for the selection and survival of B cells that differentiate either into special plasma cells or memory B cells [167].

A recent paper found that increased frequency of Tfh cells was correlated, in a cGvHD murine model, with increased germinal center B cells. Although administering a highly depletionary anti-CD20 monoclonal antibody to mice with established cGvHD resulted in peripheral B-cell depletion, B cells remained in the lung, and cGvHD was not reversed. cGvHD could be treated by eliminating production of IL-21 by donor T cells or IL-21R signaling of donor B cells. Indeed, blocking mAbs for IL-21/IL-21R, inducible T-cell costimulator (ICOS)/ICOS ligand, and CD40L/CD40 hindered germinal center formation and cGvHD. Development of cGvHD was also dependent upon T cells expressing the chemokine receptor CXCR5 to facilitate T-cell trafficking to secondary lymphoid organ follicles [168].

I.3.2.2.2 Regulatory T cells

T_{regs} (defined as $CD4^+ CD25^+ FoxP3^+$ T cells) are a functionally distinct T-cell subset which plays an important role in peripheral tolerance and homeostasis by suppressing aberrant and excessive immune responses which would be harmful for the host [169]. T_{regs} express the transcription factor Forkhead Box P3 (FoxP3), which is the master regulator for T_{reg} development and function and is considered lineage-specific. Indeed, mutations in the FoxP3 gene, which leads to a lack of (or dysfunctional) T_{regs}, are associated with lethal immunity both in mice (Surfy mice) and in humans (immunodysregulation polyenendocrinopathy enteropathy X-linked (IPEX) syndrome). The main population of T_{regs} is generated in the thymus (tT_{regs}). However, T_{regs} can also be generated in the periphery (pT_{regs}) or in vitro from naive $CD4^+ CD25^-$ T cells by T cell-receptor stimulation along with TGF-β, IL-10 or retinoic acid (iT_{regs}) [170]. Although forced expression of FoxP3 in T convs confers them a suppressive activity [171], FoxP3 expression *per se* is not sufficient for stably maintaining T_{reg} phenotype and suppressive function [172]. Indeed, T_{reg}-specific epigenetic changes (such as DNA hypomethylation) are also involved in controlling the expression of some T_{reg} function-associated molecules such as IL-2Rα, CTLA4, or FoxP3 itself [172]. Among these epigenetic changes, Foxp3 intron 1 (FoxP3i1; corresponding to FoxP3 conserved noncoding sequence 2 (CNS2)) is unmethylated in T_{regs} while heavily methylated in conventional T cells, and greatly contributes to the stability of FoxP3 expression as well as T_{reg} phenotype and functions [172].

Five mechanisms have been proposed for the control of immune responses by T_{regs} (***Figure 4; p.29***): (i) modulation of APC activity through T_{reg} engagement of co-stimulatory receptors on the surface of APC, leading to weak or abrogated signals from APC to naive/effector cells; (ii) T_{reg} secretion of cytokines, such as IL10 and TGF-β, suppressing the activity of effector cells and APCs; (iii) under certain circumstances, T_{regs} could have a direct cytotoxic effect, through the production of perforin/granzyme and induction of apoptosis in effector cells; (iv) T_{regs} may also cause metabolic disruption, for example stimulating APCs to produce enzymes that consume essential amino-acids, preventing naive/effector cell proliferation, and in the presence of TGF-β may induce the expression of Foxp3 in naive cells (i.e., they become T_{regs}); (v) T_{regs} could also compete with effector cells for APC signals or cytokines, such as IL2 [173].

As mentioned above, much attention has been paid to the role of T_{regs} after allo-HSCT in the last decade. In murine experimental GvHD models, administration of high doses of T_{reg} (Tconv/T_{reg} ratio of 1 or 2) at the time of transplantation prevented aGvHD without apparently impairing GvL effects [174, 175]. Similar observations have been made in humanized mouse models [176, 177]. Further, in humans, T_{reg} infusion allowed safe injection of an otherwise lethal dose of Tconv in

the HLA-haploidentical HSCT setting [114], and prevented aGvHD in adult patients given double unrelated cord blood transplantation [113].

T_{regs} have also been extensively investigated as potential actors for cGvHD pathogenesis, [178, 179]. Some early studies found a correlation between high numbers of T_{regs} in blood and a reduced incidence of cGvHD [179, 180], while others reports showed the reverse [181, 182]. However, the role of T_{regs} was later established in a murine model of cGvHD where infusion of donor splenic activated ($CD103^{+}$) T_{regs} prevented cGvHD [183] and in human studies where it was demonstrated that administration of low doses of IL-2 increased T_{reg} numbers and improved cGvHD in a cohort of patients with steroid-refractory cGvHD [184-186].

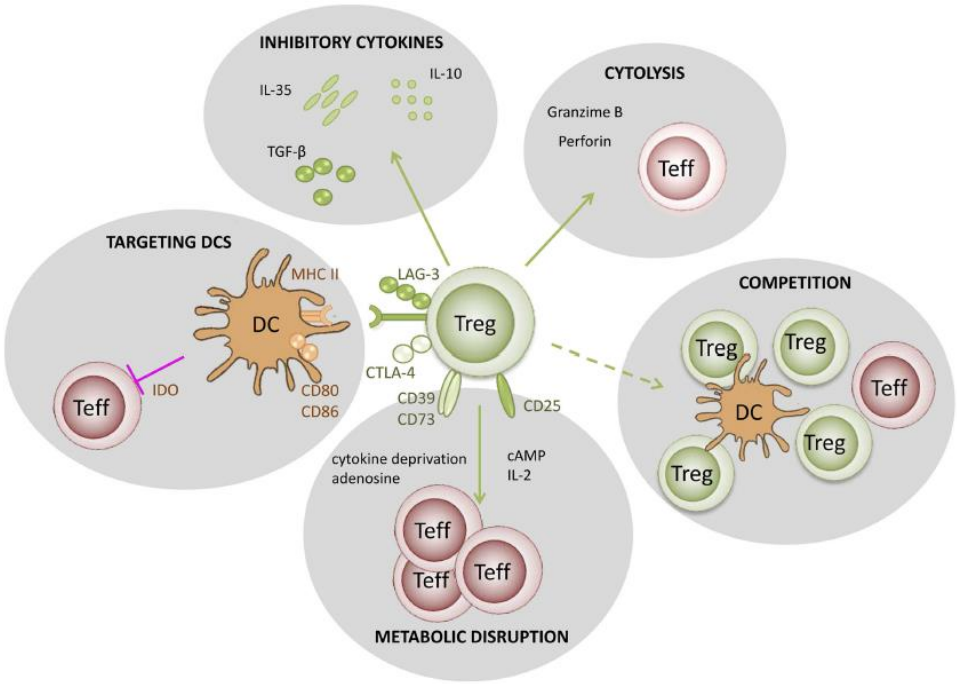

Figure 4: T_{reg}-mediated suppression mechanisms (Caridade et al., 2013 [173]) (1) Targeting DCs – modulation of antigen-presenting cell activity through T_{reg} engagement of co-stimulatory receptors on the DC surface, leading to weak or abrogated signals to naive/effector T cells; (2) Metabolic disruption – includes cytokine deprivation, cyclic AMP-mediated inhibition, and adenosine receptor (A2A)-mediated immunosuppression; (3) Competition – for critical cytokines, such as IL-2, or direct disruption of effector cell engagement with APCs; (4) Cytolysis – direct cytotoxic effect through the production of Granzyme B and Perforin and

consequent apoptosis of effector T cells or APCs; (5) Production of inhibitory cytokines – including IL-10, IL-35, and TGF-β.

I.3.2.2.3 B Cells

B cells are also involved in the pathogenesis of cGvHD; both recipient B cells by priming donor T cells, as well as donor B cells by producing allo- or autoantibodies (including antibodies agonist to the PDGF-receptor (PDGF-r) that could directly stimulate fibrosis [187] or autoantibodies directed against Y chromosome-encoded miHAs in male patients given grafts from female donor) and by secreting pro-inflammatory cytokines [188]. The production of autoantibodies in cGvHD is thought to be due to the large amounts of BAFF present after transplantation in patients with cGvHD. Indeed, high BAFF levels can promote expansion of auto-reactive B cells [189, 190].

Direct demonstration of the role of B cells in GvHD pathogenesis was reported in murine studies. *Johnston* et al. demonstrated that anti-CD20 mAb treatment the day after HSCT or before the appearance of serum autoantibodies was able to prevent induction of cGvHD by depleting donor B cells and protecting the host thymus in two cGvHD murine models. In addition, the anti-CD20 mAb treatment did not interfere with the GvL effect [191]. Moreover, blockade of CD40 ligand, blockade of T-cell co-stimulation by CTLA4, stimulation of the TNF receptor superfamily member 4-1BB, and skewing towards a Th1 predominant phenotype with administration of IL-12 have demonstrated an inhibition of antibody production [192-194]. Finally, in the clinical setting, it was demonstrated that *in vivo* depletion of B cells by rituximab, a chimeric murine/human monoclonal antibody directed against the CD20 antigen (exclusively expressed on B cells) reduced the incidence of cGvHD [195-198], while rituximab administration also improved cGvHD in approximately 2/3 of patients with steroid-refractory cGvHD [199].

B cell importance for cGvHD pathogenesis was also highlighted by the spleen tyrosine kinase (Syk) in a murine cGvHD model, dependent on antibody and germinal center B cells. Disease progression depends on Syk activation and was mandatory in donor B cells, but not T cells. *In vivo* BM Syk deletion was effective in treating established cGvHD, as well as fostamatinib (Syk inhibitor), by induction of B cell apoptosis [200].

Moreover, ibrutinib, an inhibitor of Bruton's tyrosine kinase and IL-2 inducible T-cell kinase that targets Th2 cells and B cells, was recently demonstrated to ameliorate ongoing murine cGvHD. In a sclerodermatous cGvHD (scl-cGvHD) model, improved survival and ameliorated clinical and pathological manifestations were observed in ibrutinib-treated mice. Similar observations were made in an alloantibody-driven cGvHD model, in which ibrutinib treatment improved pulmonary function.

Furthermore, decreased tissue immunoglobulin deposition was also observed in animals treated with ibrutinib. Finally, T- and B-cell activation was reduced in cGvHD patients receiving ibrutinib [201].

However, a recent publication demonstrated the role of B regulatory cells in cGvHD prevention. Indeed, $CD19^{-/-}$ donor mice induced more severe scl-GvHD than wild-type donors. $CD19^{-/-}$ recipients experienced no significant difference compared with wild-type recipients. Increased GvHD severity could be explained by higher expansion of splenic IL-6–producing monocytes/macrophages, cytotoxic $CD8^{+}$ T cells, and Th1 cells during the early stage of disease and increased infiltration of T cells, TGF-β–producing monocytes/macrophages, and Th2 cells into the skin in the later stage of Scl-cGvHD. Furthermore, IL-10–producing regulatory B cells were not reconstituted in recipients receiving $CD19^{-/-}$ cells, indicating that donor-derived B regulatory cells have a suppressive role in scl-cGvHD development [202]. Recently, *de Masson et al.* found a relation between decreased B regulatory cell frequencies and cGvHD severity. An impaired ability of B cells to produce IL-10 was found in patients with active cGvHD. In vitro plasmablast differentiation increased the frequency of IL-10-producing B cells. They also observed that recipients with cGvHD had fewer $CD24^{high}CD27^{+}$ B cells and IL-10-producing $CD24^{high}$ $CD27^{+}$ B cells, but increased plasmablast frequencies and decreased IL-10-producing plasmablasts [203].

I.3.2.2.4 Profibrotic-Inflammatory Cytokines

I.3.2.2.4.1 Transforming Growth Factor- β

TGF-β is a pleiotropic cytokine, which exists in three isoforms (TGF-β1, TGF-β2 and TGF-β3) in mammals. TGF-β affects multiple cell lineages by promoting or opposing their differentiation, survival and proliferation. It is also an important cytokine for the control of apoptosis, angiogenesis, wound healing, tumor biology, and inflammation/immune regulation. The later is best demonstrated by the observation that TGF-β1-deficient mice succumb 3-4 weeks after birth from a wasting syndrome including multifocal, mixed inflammatory cell response and tissue necrosis, leading to organ failure [204]. Further, TGF-β plays an important role in the generation of iT_{regs} [205], and, in combination with IL-6, induces Th17 cells [206].

As mentioned above, fibrosis is a hallmark of human cGvHD. TGF-β is secreted by activated macrophages in an inactive form bound to the latency-associated protein (LAP). When the complex TGF-β-LAP is cleaved, TGF-β binds to its receptor (TGF-β receptor; TGF-β-r) on fibroblasts. This ligand-receptor interaction induces a cascade signaling activation pathway mediated by the SMAD proteins, and results in collagen synthesis and fibrosis [207]. Several studies suggest a primordial role of TGF-β in cGvHD-associated fibrosis. First, it has been observed in a mouse model of

scl-cGvHD (B10.D2 → Balb/c, *vide infra*) that TGF-β neutralization decreased cGvHD and prevented skin and lung fibrosis [208-210]. Similar observations were made with systemic administration of LAP [209, 211, 212]. Further, high TGF-β1 levels have been observed in patients with cGvHD [213]. However, in contrast to what is seen in cGvHD, TGF-β blockade exacerbates aGvHD, demonstrating the complex role of TGF-β in GvHD pathogenesis [210].

Imatinib, a tyrosine kinase inhibitor, which inhibits c-Abl, prevents production, as well as mRNA expression, of collagen (COL) 1A1, COL1A2 and fibronectin by PDGF-β and TGF-β-stimulated fibroblasts [214]. While blockade of TGF-β fibrogenesis is explained by inhibition of the TGF-β-r, it seems that c-Abl is an important cellular mediator of TGF-β-mediated fibrosis [215]. Indeed, c-Abl is implicated in phosphorylation of Smad-1, resulting in its activation, which in turn upregulates the connective tissue growth factor (CCN2) [216, 217]. Moreover, c-Abl activates also the protein kinase C (PKC)-δ, which in turn decreases the binding affinity of the transcription factor Fli 1 to the promoter of the COL1A1 gene [218]. Finally, c-Abl is also implicated in the reduction of miR-29, an anti-fibrotic microRNA, in fibroblasts [219]. These signaling pathways are thus important regulators of fibrosis.

I.3.2.2.4.2 Platelet-Derived Growth Factor

PDGF plays an important role for the maintenance of connective tissues in adults and contributes to the development of the embryo. Deregulation of its signaling pathway has been observed in atherosclerosis, pulmonary hypertension and fibrosis [220]. PDGF-r signaling pathway includes a variety of mediators. Upon engagement of PDGF-r by stimulatory antibodies, the signaling cascade composed of Ha-Ras and extracellular-signal-regulated kinases 1 and 2 (ERK 1/2) will result in collagen gene expression and production of reactive oxygen species (ROS). Elevated levels of ROS will in turn stabilize the HA-Ras pathway, thus maintaining this signaling cascade [187].

Scleroderma and systemic sclerosis are autoimmune diseases characterized by fibrosis and vasculopathy. Recent studies have demonstrated a crucial role of PDGF on fibrosis (and perhaps also on vasculopathy) in systemic sclerosis. Specifically, systemic sclerosis patients express high levels of PDGF and PDGF-r in skin lesions and bronchoalveolar lavage [221-223]. In murine models, PDGF transgenic overexpression induces tissue fibrosis [224-226]. Importantly, autoantibodies with agonist function to the PDGF-r have been demonstrated in patients with systemic sclerosis [187].

As mentioned above, cGvHD share many features with systemic sclerosis. Interestingly, agonist autoantibodies to PDFG-r have also been demonstrated in patients with extensive cGvHD, suggesting that the PDFG-r pathway plays also a role in cGvHD pathogenesis [227].

I.3.2.3 Murine models of chronic Graft-versus-Host Disease

While murine models of aGvHD have provided a clear vision of its pathophysiology, the understanding of the pathophysiology of cGvHD has been limited by the lack of animal models that encompassed all features of the human disease. Nevertheless, murine models of cGvHD have provided useful information regarding cGvHD pathophysiology, and new potential therapeutic molecules.

Current murine models of cGvHD (***Table 2***) can be divided in three categories: sclerodermatous (pro-fibrotic) models, autoantibody-mediated (lupus-like) models, and thymic function defective models. This classification is based on the phenotype and immunologic mechanisms resulting in cGvHD development.

Table 2. *Establishment of Selected Murine Models of cGVHD*

cGVHD Model	Donor -> Recipient Strains	Cells (Cell Dose)	Radiation Dose (cGy)	Clinical Phenotype[a]
SLE-cGVHD	*B6 -> (B6 × DBA2)F1*	*Spl (8×10^7-1×10^8) only*	*None*	*Auto Ab/ICG*
	B6 -> (B6 × DBA2)F1	TCD BM (1×10^7) + Spl (5×10^6)	900	IPS
	B6 -> (B6 × BALB/c)F1	Spl (6×10^7) only	None	aGVHD ->
	bm12[b] -> B6	Spl (1×10^8) only	None	cGVHD
	DBA2 -> BALB/c	Spl (5×10^7) only	650	Auto Ab/nephritis Auto Ab/Scl
Scl-cGVHD	*B10.D2 -> BALB/c*	*±TCD BM ($1 \times 10^6 - 1 \times 10^7$) + Spl ($6 \times 10^6 - 1 \times 10^8$)*	*700-1000*	*Scl/fibrosis[c]*
	B10.D2 -> BALB/c		600	Scl/fibrosis
	B10.D2 -> BALB/c	Spl ($2.5 \times 10^7 - 1 \times 10^8$) only	850	Parotid Dysfxn.
	[C3H.SW->B6]CD4 -> B6	Spl (2.5×10^7) only	1000	Scl
	B6 -> CB6F1	TCD C3H.SW BM (5×10^6) + CD4 (3×10^5)[d]	1100	Scl
	DBA2 -> BALB/c	TCD BM (5×10^6) + Spl (3×10^6) Spl (5×10^7) only	650	Auto Ab/Scl
cGVHD because of thymus dysfunction	B6-H2-Ab1$^{-/-}$-> C3H/HeN	TCD BM (5×10^6)	1300	Scl/fibrosis

Table 2: cGvHD murine models (from Chu and Gress, 2008)

I.3.2.3.1 Sclerodermatous models

Scl-cGvHD models are characterized by progressive fibrosis in the dermis, and sometimes in the lungs, liver and salivary glands [209, 228-231]. In these models, donor mature splenocyte infusion in addition to donor bone marrow in conditioned recipient mice results in full donor chimerism. The cGvHD phenotype resembles human scleroderma, as observed in up to 15% of patients with cGvHD [232]. However, the time necessary to develop fibrosis is faster than for the human disease since in the mouse model, cGvHD begins within 30 days post-transplantation while in humans it can take months to years. The B10.D2 → BALB/c

is the most common strain combination used for scl-cGvHD models [228, 233]. It requires total body irradiation as conditioning regimen. In this model, both the irradiation dose and the dose of transplanted spleen cells affect disease severity [228].

The main advantage of this model is the MHC compatibility between donor and recipient strains. Indeed, MHCs are matched but the donor and recipient differ in numerous miHAs. The pathophysiologic mechanism involved in B10.D2 → BALB/c model is well-described (***Figure 5; p.35***). The pre-transplant conditioning regimen induces tissue damage, resulting in the release of inflammatory signals as early as 7 days post-transplantation. These inflammatory signals include chemokines, such as MCP-1, MIP-1α and RANTES [234, 235]. These chemotactic molecules then induce infiltration of the skin by donor-derived monocytes, activated macrophages and T cells and increased expression of adhesion molecules (VCAM-1, ICAM-1) in target organs that participate in cGvHD [236-238].

Interestingly, both donor and recipient APCs are capable of initiating cGvHD in that model. Specifically, while both donor and recipient APCs are sufficient to induce skin cGvHD, donor APCs were found to be the primary inducer of gut cGvHD [239]. Further, while CD80/86 costimulation is sufficient to induce skin cGvHD, both CD80/86 and CD40 costimulation are necessary for the initiation of gut cGvHD [239]. These data demonstrate a complex interplay between donor/host APCs and donor T cells resulting in differential patterns of inflammation in the various target tissues.

As observed in the pathogenesis of human cGvHD, T cells are primordial for cGvHD induction in this murine model. Indeed, donor naive $CD4^+$ T cells alone are sufficient and necessary to induce scl-GvH reaction, while $CD8^+$ and memory $CD4^+$ T cells alone are insufficient to generate cGvHD [240-242]. Moreover, alloreactive $CD4^+$ T cells generated during the early GvHD phase and transferred into secondary hosts, are able to induce cGvHD [243].

As mentioned above, cytokines are also involved in cGvHD pathogenesis in that model. Specifically, TGF-β has been shown to be the main mediator of the fibrotic process. Furthermore, the Th1/Th2 balance tightly regulates fibrosis. Indeed, Th2 $CD4^+$ cells control positively the TGF-β-mediated fibrosis while Th1 cells inhibits this process. Other Th2 cytokines, such as IL-13 secreted by $CD4^+$ cells may also have an important role in scl-cGvHD. IL-13 may directly stimulate fibroblasts to produce collagen, or indirectly stimulate macrophages through the IL-13 receptor to produce TGF-β [207]. Confirming this hypothesis, it has been observed that IL-13 gene expression was increased in the skin of scl-cGvHD mice in the early phases of cGvHD.

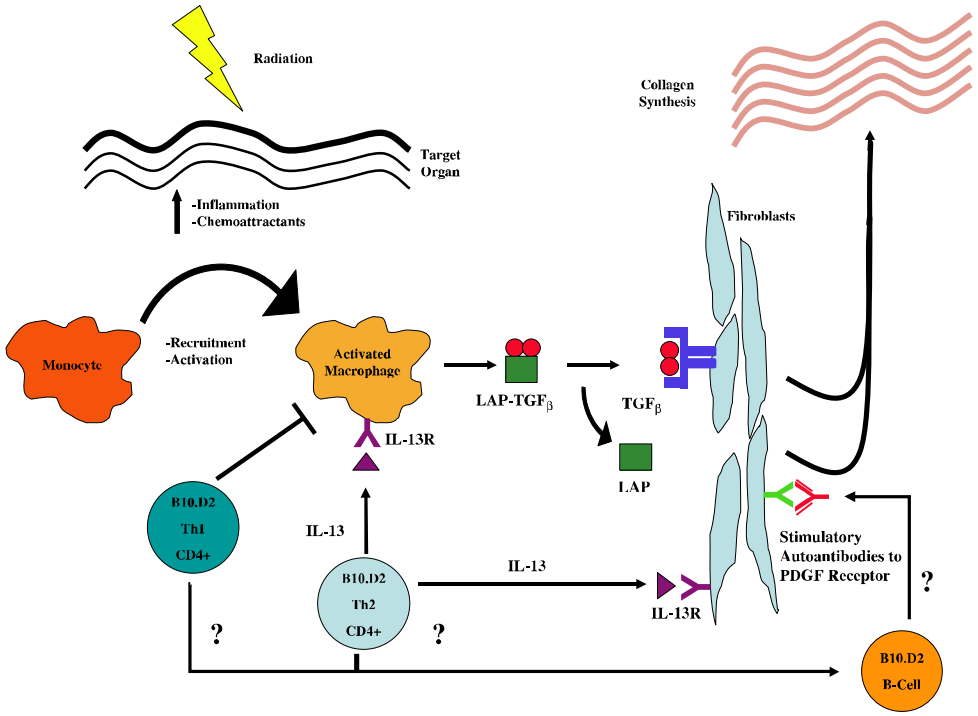

Figure 5: Pathogenesis of B10.D2 → BALB/cJ scl-cGvHD mouse model (From Chu et Gress, 2007). Illustrated are the events presumed to occur in the B10.D2 into BALB/c miHA mismatched transplant model. Tissue damage results in the upregulation of inflammatory mediators and chemoattractants in target tissues and of adhesion molecules in monocytes and donor-derived T cells. Upon activation by donor and/or host APC (not shown in the figure), $CD4^+$ T cells are recruited into target tissues and signal activated macrophages to produce TGF-β by way of IL-13 signaling. TGF-β, in turn, binds to its receptor in fibroblasts resulting in increased collagen synthesis, leading to fibrosis. In addition to stimulation of collagen by fibroblasts through TGF-β signaling, $CD4^+$ T cells can directly stimulate collagen synthesis by fibroblasts through IL-13 signaling. Clinical data demonstrating the role of stimulatory autoantibodies against the PDGF-r are also shown, but their role in murine cGvHD has not yet been demonstrated.

In addition to the pro-fibrotic pathways as a major player in scl-cGvHD pathogenesis, defects in immunoregulation have also been observed. Indeed, reconstitution of BALB/c $RAG^{-/-}$ mice with $CD4^+CD25^+$ T-cell (T_{regs})-depleted spleen cells (instead of unfractionated $CD4^+$ T cells) from B10.D2 donor mice results in a more aggressive cGvHD reaction while addition of $CD4^+CD25^+$ T cell from either donor or host in the graft decreased cGvHD [244].

In summary, the B10.D2 → BALB/c scl-cGvHD model shares many features with human scl-cGvHD, a form of cGvHD associated with a poor prognosis in humans [126, 245-247]. Importantly, mediators involved in murine scl-cGvHD seem similar to those involved in humans. Indeed, post-transplant TGF-β serum levels are increased in cGvHD patients compared to those without cGvHD [213]. Further, high levels of IL-13 have been observed in the bronchoalveolar lavage fluids of patients with bronchiolitis obliterans, suggesting a pro-fibrotic role for this cytokine during human scl-cGvHD [248]. However, there are some discrepancies between this mouse model and human disease, such as the presence of stimulating anti-PDGF-r autoantibodies in patients with extensive scl-cGvHD [227, 249] but not in the B10.D2 → BALB/c scl-cGvHD model. Further, fibrosis in murine scl-cGvHD is primarily limited to the skin, while fibrosis in cGvHD patients could be systemic or pleiotropic.

I.3.2.3.2 Autoantibody-mediated model

Models of autoantibody-mediated cGvHD are typically MHC-mismatched models, and classically involve parent-to-F1 transplants that result in mixed chimerism. The most commonly used strain combination for autoantibody-mediated model is DBA/2 donor mice into B6D2F1 recipient mice (DBA/2 → B6D2F1). Recipient mice exhibit a "cGvHD phenotype" close to what is observed in human lupus, including nephritis, biliary cirrhosis, salivary gland fibrosis, lymphadenopathy, splenomegaly, and to a lesser extend, skin fibrosis (***Figure 6; p.37***).

This model is characterized by recipient B-cell expansion. These recipient B cells act as APCs and activate $CD4^+$ T cells while polarizing them to a Th2 cytokine production [111, 192, 193, 250-255]. Th2 $CD4^+$ T cells then promote B-cell activation and production of autoantibodies [256-259]. Interestingly, shifting the cytokine balance from Th2 into Th1 induces aGvHD [260]. Finally, blockade of CD40 ligand, blockade of T-cell costimulation with CTLA4Ig, and stimulation of necrosis factor receptor superfamily member 4-1BB, all attenuate or prevent sle-cGvHD by inhibiting T-cell dependent antibody production [193, 194, 261].

Autoantibody production observed in this model shares some similarities to what is observed in patients with cGvHD. However, this model has many discrepancies with human cGvHD. These discrepancies include 1) the absence of conditioning regimen in the mouse model, 2) the absence of infusion of hematopoietic stem cells, 3) a lower diversity of autoantibodies than in humans, and 4) the development of severe nephritis in mice (which is uncommon in cGvHD patients) [262, 263].

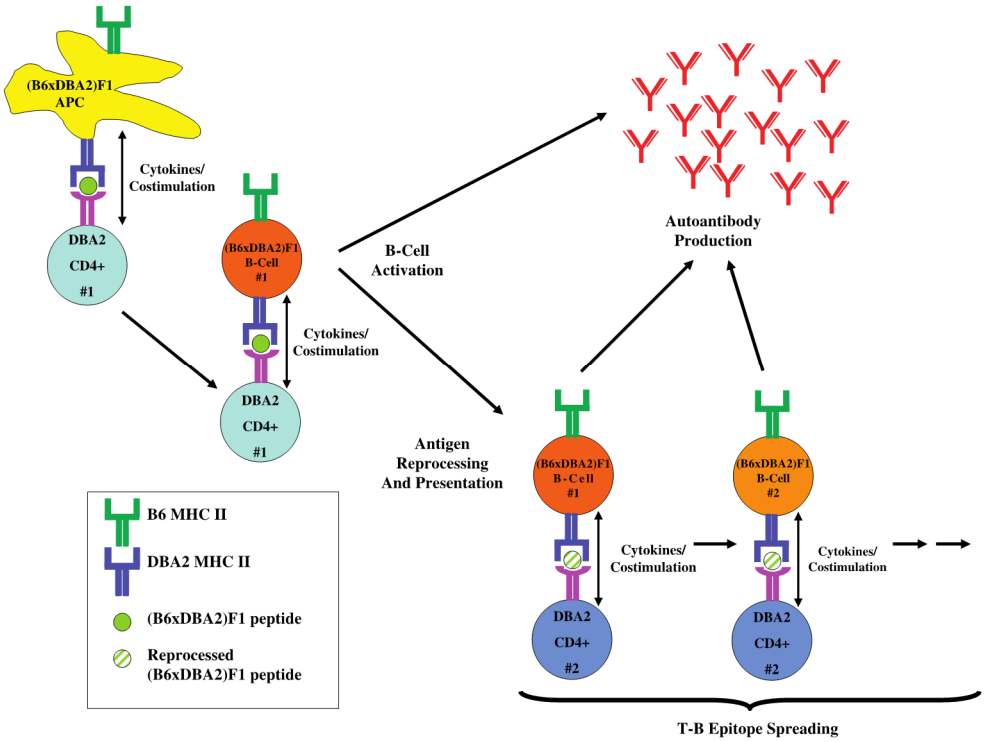

Figure 6: Pathogenesis of the DBA/2 → B6D2F1 sle-cGvHD mouse model (From Chu et Gress, 2007). Illustrated are the events presumed to occur in the DBA2 into (B6xDBA2)F1 model of systemic lupus erythematosus (Sle)-cGvHD. DBA2 $CD4^+$ T-cells are stimulated by host (B6xDBA2)F1 APC through interactions between the peptide presented in the context of host class II MHC. Activation of $CD4^+$ T cells in turn stimulates host B cells to produce autoantibodies. In addition, B cells could stimulate additional donor $CD4^+$ T cells through antigen reprocessing and presentation in the context of its class II MHC. In this way, the generation of autoantibodies against a progressively wider range of epitopes is perpetuated.

Despite these limitations, the sle-cGvHD mouse model has contributed to the understanding of the pathogenesis of human cGvHD by highlighting the role of B lymphocytes.

I.3.2.3.3 Assembling scleroderma and autoantibody production in a single mouse model

Attempts to combine in one single mouse model features of both sclerosis and of autoantibody production have been made with the aim of more accurately reproduce features encountered in human cGvHD. In particular, *Zhang* et al. combined the B10.D2 → BALB/c model with the DBA/2 → B6D2F1 by infusing

splenocytes from DBA/2 into lethally irradiated BALB/c reicipient mice. This resulted into both a lupus-like and a scleroderma-like phenotype [183]. Specifically, recipient mice developed collagen deposition leading to skin fibrosis, proteinuria and autoantibodies directed against double-stranded DNA with immune complex deposits. Further, unlike what is observed in the classical sle-model in which recipient B cells but not donor B cells are involved, donor B cells were clearly involved in the mechanisms of this new cGvHD mouse model. Finally, T_{regs} seemed also important since infusion of these cells decreased the severity of cGvHD [183].

I.3.2.3.4 Defective thymic function model

Recently, a new mouse model of cGvHD has been developed (transgenic transplant H2-Ab1$^{-/-}$ → C3H/HeN mice), based on defective thymic activity. This model consists in the infusion of T-cell depleted bone marrow from MHC II-deficient C57/Bl6 mice (H2-Ab1$^{-/-}$ H2^{b}) in lethally irradiated C3H/HeN (H2^{k}) recipient mice [150]. Recipient mice developed a cGvHD phenotype mimicking human cGvHD, because of thymic function impairment due to the absence of MHC II antigens on host thymic DCs but not on thymic epithelial cells. The clinical symptoms include skin fibrosis, bile duct loss, periportal fibrosis in the liver, salivary gland fibrosis, and cytopenias. The recipient mice developed cGvHD phenotype within 6 weeks post-transplantation and mortality was very high since less than 20% of recipient mice survived beyond day 100. Interestingly, thymic T_{reg} production is not affected in that model, but thymic T_{regs} failed to prevent cGvHD progression (***Figure 7; p.39***) [150].

The H2-Ab1$^{-/-}$ → C3H/HeN mouse model is the only model demonstra□ng a direct relationship between aberrant thymic function and development of cGvHD. It also demonstrates the central role of APCs in establishment of central tolerance and peripheral stimulation of donor T cells against host antigens.

In summary, in addition to the fact that this mouse model is the first to reproduce many features encountered in human cGvHD, it also supports the concept that dysfunctional thymic negative selection, caused by donor alloreactive T cells, could be one of the mechanisms for cGvHD development in humans.

I.3.2.3.5 Bronchiolitis obliterans cGvHD murine model

Recently, *Srinivasan* et al. proposed a new murine model of cGvHD. In this model, mice are conditioned on D-3 and D-2 with cyclophosphamide (120 mg/Kg/Day i.p.) followed by total body irradiation (7.5 Gy) on D-1. On the day of transplantion (D0), recipients received 10x10^{6} bone marrow cells with or without allogeneic spleen cells (0.75 to 1x10^{6}) or purified T cells (0.1 to 0.33x10^{6}) [264].

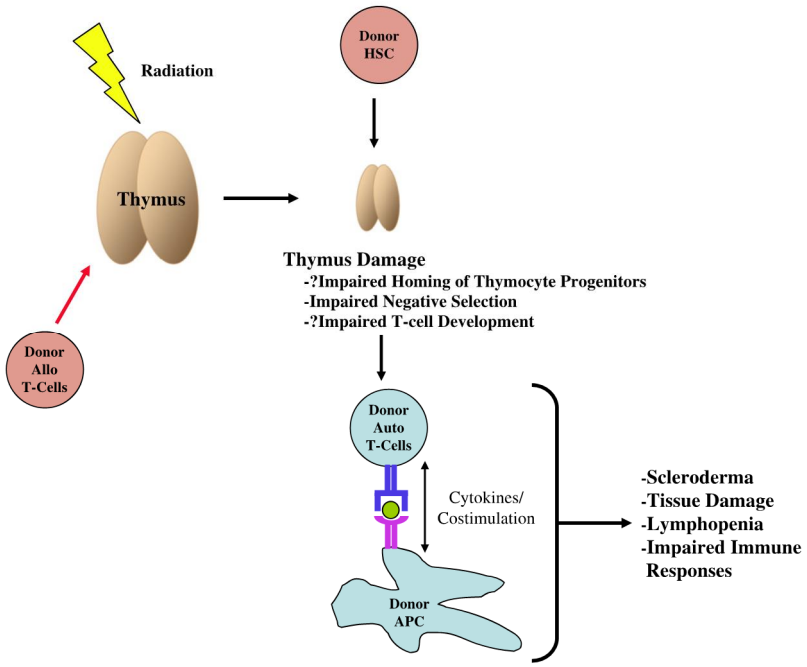

Figure 7: Pathogenesis of the C57/BL6 (H2-Ab1$^{-/-}$ H2^{b}) → C3H/HeN (H2^{k}) defective thymic function cGvHD mouse model (From Chu et Gress, 2007). The thymus is critical for central tolerance. Damage to the thymus by radiation and/or infiltration of donor alloreactive T cells results in thymic damage that leads to impaired negative selection and T-cell development as modeled by transplantation of thymic DC lacking class II MHC. Impaired negative selection leads to the development of host-derived donor-reactive T cells, which, upon activation with donor-derived APC, lead to cGvHD.

The use of cyclophosphamide exacerbates glutathione redox reaction in the lung induced by total body irradiation [265]. In synergy with injection of sublethal doses of donor T cells, these mechanisms favor the development of cGvHD. When the model was first published in 2007, the only reported manifestations were tissue fibrosis in the lungs [266], resulting in the development of bronchiolitis obliterans, which is the typical manifestation of lung cGvHD [127]. On further examination, these mice also developed pathologic manifestations in several other organs including the liver, tongue, thymus, colon, and spleen [264].

Donor CD4^{+} T and B220^{+} B cells were reported to play a major role in the development of cGvHD in target organs, while CD8^{+} T cells do not seem to play a causative role in the development of bronchiolitis obliterans in this model. Moreover, antibody deposition was found in target areas of lung and liver (the

paper focused on these two organs) with a presence of these antibodies in serum. However, the origin of these antibodies remains unclear. More importantly, this model seems to indicate that B cells from germinal centers of secondary lymphoid organs seem to be critical for the development of cGvHD [264]. Follicular B cells express LT-αβ, whereas follicular DCs express LTβR. Inhibition of this interaction by use of the LTβR-Ig fusion protein prevents germinal center formation [267]. Treatment with LTβR-Ig had a direct effect on the symptoms of cGvHD, at least in part by blocking germinal center formation. These effects included an alleviation of pulmonary dysfunction. There was also a decrease in tissue-specific Ig levels in the sera [264]. Finally, LTβR-Ig blocked also LIGHT, which is expressed on activated T cells and on B cells [267]. This group has also demonstrated a role for IL-21, CD40L and ICOS in this model [168].

I.4 Tyrosine kinase inhibitors

I.4.1 Introduction

Tyrosine Kinase inhibitors (TKIs) are small molecules that inhibit tyrosine kinases. Imatinib Mesylate (STI571; Glivec, Novartis, Basel, Switzerland) has been developed as a selective PDGF-r and Break Cluster Region-Abelson Fusion Gene (BCR-ABL) inhibitor [268, 269]. It has been the first-line treatment for Philadelphia chromosome-positive chronic myeloid leukemia (CML) and is increasingly used in allogeneic transplantation as prevention/treatment of relapse in patients with BCR/ABL^+ hematological malignancies. However, imatinib not only inhibits BCR-ABL but also the tyrosine kinases c-Abl and c-Kit [270, 271]. The use of imatinib in the clinic is generally safe and overall side effects are manageable. However, patients on imatinib may develop mutations that limit its efficacy and cause leukemic relapses [272].

Nilotinib (AMN-107; Tasigna, Novartis) is a second generation TKI (such as dasatinib and bosutinib). It was developed by direct modification of the chemical structure of imatinib. Nilotinib is a more potent and more selective inhibitor of the BCR-ABL protein compared to imatinib. Moreover, it inhibits the majority of leukemic cells expressing BCR/ABL^+ imatinib-resistant mutations with the exception of the T315i mutation [273].

I.4.2 TKIs in cGvHD

TKIs have immune-modulatory effects on both innate and adaptive immune responses. Indeed, c-Abl is an important cellular mediator for B-cell and T-cell responses. Deficient c-Abl signaling in T cells results in decreased production of IL-2 and cell proliferation [274]. Further, c-Abl-deficiency in B cells decreases the phosphorylation of the B-cell receptor and B-cell proliferation. Consequently, ABL

knocked-out mice have altered B-cell and T-cell responses and development [274-276].

I.4.2.1 TKIs and T cells

Tyrosine kinases (including c-Abl, c-kit and LCK) play a crucial role in TCR signaling. Specifically, as mentioned above, the ablm1 mouse (which contains a mutated src kinase docking site on c-abl,) has a reduction in peripheral mature T cells and a diminished T-cell progenitor cell number. Interestingly, it must be noted that a similar phenotype is also induced by imatinib treatment of wild-type adult mice [277]. In addition, c-kit has been shown to be important for T-cell development since adult c-kit^{null} mice have a significant reduction in thymic T-cell progenitor cells [278]. Finally, imatinib also inhibits LCK, a tyrosine kinase necessary for activation and proliferation of T cells. Indeed, engagement of the TCR activates LCK, inducing phosphorylation of the intracytoplasmic portion of the TCR; which, in turn, activates several signaling pathways required for T-cell activation and proliferation [279-281].

Confirming observations in mouse models, imatinib also inhibits the proliferation and activation of human T cells. Indeed, T-cell proliferation could also be decreased by TKIs. This observation could be explained by cell cycle arrest in phase G_1/G_0, resulting in DNA synthesis inhibition both *in vitro* and *in vivo* through blockade of c-Kit and c-Abl. Furthermore, this effect is dose-dependent and does not induce apoptosis [279, 280]. Finally, imatinib also inhibits IL-2, TNF-α and IFN-γ secretion from both healthy donors and patients being treated for CML [280, 282].

I.4.2.2 TKIs and T_{reg} cells

The role of TKIs in T_{reg} regulation is still incompletely understood. However, some *in vitro* studies demonstrate an impaired immunosuppressive function of T_{reg} cells cultured with imatinib at therapeutic concentrations [283]. This decreased suppressive capacity was accompanied by lower FoxP3 expression. However, proliferation was not significantly affected. These experimental observations could be explained by a significant reduction in the activation of STAT3 and STAT5 transcription factors and phosphorylation of ZAP70 and LAT [283].

These preliminary data support the hypothesis that TKIs are good candidates for targeting T_{regs} in cancer immunotherapy or immune diseases by modulating T_{reg} suppressive function. However, in the context of allo-HSCT, this T_{reg} function impairment might be a problem for GvHD treatment with imatinib.

I.4.2.3 TKIs, B cells and dendritic cells

Inhibition of BCR signaling and IgM production by imatinib has been observed *in vivo*. These inhibitions could be explained by a decreased c-Abl kinase activity by imatinib at low therapeutic concentrations. These observations were confirmed by clinical observations since CML patients treated with imatinib experienced hypogammaglobulinemia [284, 285].

Further, imatinib could have inhibitory effects on DC activation, since the Src kinase family is important for this process [286, 287]. Moreover, it seems also to affect the number and function of this immune cell subpopulation. Differentiation of primitive human $CD34^+$ progenitors and monocytes into dendritic cells is also inhibited but only at high concentrations [288, 289]. This results is hampering the induction of primary cytotoxic $CD8^+$ cells due to low levels of co-stimulatory molecules at the surface of DCs. However, contradictory results have been published. Indeed, DCs treated with imatinib can also result in enhancement of APC function [290, 291].

I.4.2.4 Anti-inflammatory and anti-fibrotic effects of TKIs

In addition to its impact on adaptative immunity, imatinib can also inhibit innate and inflammatory responses. Specifically, imatinib can inhibit macrophage activation and TNF-α production [292]. Furthermore, it also prevents mast cell and pro-inflammatory cytokine production, as well as fibroblast activation and proliferation (through PDGF-r inhibition, as mentioned above) [292].

The anti-fibrotic effects of imatinib were first demonstrated in a mouse model of bleomycin-induced dermal fibrosis. It was demonstrated that imatinib decreased dermal thickness, number of myofibroblasts, and amount of collagen in the skin [214, 293]. Similar observations were obtained with nilotinib, the second generation TKI, at therapeutically relevant doses reached in humans [294]. The anti-fibrotic effects were also confirmed in animal models of pulmonary, renal, cardiac and liver fibrosis [215, 295-297].

Further, a recent study by Zerr et al. assessed the impact of imatinib or nilotinib on cGvHD in the scl-cGvHD mouse model. Interestingly, the authors observed that both imatinib (150 mg/Kg once a day) and nilotinib (37.5 mg/Kg b.i.d) decreased the aberrant activation of c-Abl and PDGF-r and prevented experimental cGvHD. Further, clinical manifestations (weight loss, alopecia, skin ulcers) and histologic features (dermal thickening and accumulation of collagen) were significantly decreased in mice given imatinib or nilotinib than in control mice. Remarkably, both TKIs were also effective in the treatment of established murine cGvHD. From this pre-clinical study, it seems that the combined inhibition of c-Abl and PDGF-r is

effective for both prevention and treatment of scl-cGvHD with imatinib or nilotinib [298].

Based on the anti-fibrotic potential of imatinib and nilotinib, a number of phase II clinical studies assessed the impact of imatinib on fibrosis in patients with systemic sclerosis and mixed connective tissue diseases. Specifically, 30 patients affected by systemic sclerosis were treated with imatinib 400 mg/day [299, 300]. After twelve months of treatment, imatinib significantly improved morphology, fibrosis and thickness of the skin. No differences were observed in lung disease, which remained stable. In agreement with the absence of responses in lung fibrosis observed in the above study, another recent report demonstrated that imatinib failed to improve idiopathic pulmonary fibrosis in a cohort of 119 patients [299, 300].

I.4.2.5 TKIs for cGvHD: clinical data

The first case report supporting the effects of imatinib in cGvHD was published in 2006. A 33-year-old CML patient received allo-HSCT. She develops steroid-refractory cGvHD with severe lung involvement. As she relapsed, imatinib treatment was started and her pulmonary cGvHD improved after 2 months [301]. Subsequently, in 2008, another publication reported data from two patients with scl-cGvHD who were treated with imatinib. Remarkably, both patients experienced improvement in scleroderma symptoms within the first 3 months [302].

Based on these case reports, a phase I-II of 19 patients suffering from steroid-refractory cGvHD with fibrotic features was initiated. All patients had a long history of cGvHD with multiorgan involvement including the skin. Imatinib was well tolerated at the dose of 100 mg/day (increased at 200 mg if initial dose was well tolerated) [303]. Interestingly, fibrosis significantly improved within the first 6 months in 15 of 19 patients, with 7 patients reaching CR (of the skin or of all signs of GvHD) and 8 others PR.

In 2008, a multicenter center phase I-II study of treatment of scl-cGvHD with low dose imatinib (initial dose of 100 mg/day, increased to 200 mg/day and if no response occurred the dose was further increased up to 400 mg/day) was launched by *Oliveiri* et al. on a larger population of patients. Response in this study was evaluated by using the more stringent response criteria published by the NIH Consensus Conference for cGvHD. Primary endpoints included safety and efficacy of low dose imatinib. Intermediate analysis of the first 24 patients confirmed that imatinib was safe and well tolerated. However, the response rate was lower in that study than in the first study. Differences in response rates between these studies could be partly explained by the use of more stringent response criteria, or by the inclusion of older patients with less fibrotic features in the latest study [304, 305].

Nevertheless, combination of these two studies confirmed the safety of imatinib for cGvHD and no toxic deaths were observed. Hematological toxicity was limited and mainly consisted in anemia, while extra-hematological toxicities included mainly infections and fluid retention. The best responses were achieved in patients with skin and gastro-intestinal involvement. Lung GvHD was poorly improved since only 29% of them achieved a PR.

Another study confirmed the efficacy of imatinib (400 mg/day) in 14 patients with skin fibrosis with imatinib started at a median of 44 months after allo-HSCT. Twelve patients responded to imatinib (including 7 PR and 5 minor responses (MR)), while the dose of corticosteroids was decreased in 4 patients due to drug intolerance [306].

In contrast to the encouraging results described above, *Stadler* et al. recently reported limited efficacy of imatinib in 9 cGvHD patients. Patients were affected by skin, mucosal, visceral, and/or fasciitic GvHD in addition to pulmonary cGvHD. Patients failed to respond to primary treatments consisting in extensive combination therapies with steroids, calcineurin inhibitors, MMF, and/or extracorporeal photopheresis. Imatinib was started at a dose of 100 mg/day and increased up to 400 mg/day as tolerated. After 4 months of treatment and using the NIH response criteria, only 1 patient recovered from severe to moderate cGvHD, only 2 patients reduced steroids while 5 patients experienced no change and 2 died due to cGvHD progression, contrasting with the result found by *Oliveiri* et al. [307].

Furthermore, a retrospective study including data from 39 patients with steroid-resistant sclerodermatous grade II or grade III skin cGvHD according to the NIH grading reported less convicting results. Specifically, imatinib failed to improve skin sclerosis in 70% of patients (stability in 31% of patients, and even worsening in 39%). Only 1 patient (2%) achieved CR, while 9 (23%) had improvement and 2 (5%) had improvement and secondary worsening after initial remission. Further, imatinib was discontinued in 22 patients (56%) due to serious adverse events (fluid retention and cytopenias) [308].

From these studies, one could conclude that although the use of TKIs for cGvHD seems encouraging, less than half of the patients seems to benefit from the treatment (***Table 3; p.45***).

	Olivieri et al. [303, 305]	Magro et al. [306]	Stadler et al. [307]	de Masson et al. [308]
Patients	40	14	9	39
Imatinib dose	100–400 mg	400 mg	100-200 mg	100-600 mg
Response	14 PR ; 4 MR	7 PR ; 5 MR	2 PR	1 CR ; 11 PR
OS (median follow-up)	72% (26 months)	92% (56 months)	77% (4 months)	/

Table 3: Most important studies evaluating imatinib for cGvHD in the clinical setting. CR = Complete response; PR = Partial response, MR = Minor response.

Nilotinib, a second generation TKI, has a better safety profile than imatinib and an equivalent PDGF-r inhibitory profile. These observations prompted Olivieri *et al.* to launch a phase I-II study aimed at assessing the safety and efficacy of nilotinib 200 mg/day (up to 600 mg/day) in 12 patients with refractory cGvHD [309]. Two patients stopped nilotinib within 30 days (cGvHD progression, asymptomatic hypertransaminasemia). Severe adverse events were reported in 3 other patients (extramedullary relapse of acute leukemia, transient cGvHD flare, late cGvHD progression). Extrahematological toxicities included headache, nausea, pruritus, cramps, asthenia, constipation, while the main hematological abnormalities were anemia, neutropenia and lymphocyte count increase. After 3 months of treatment with nilotinib, 6 patients achieved an objective improvement, 4 had a stable disease and the two others died. These preliminary data suggest that, like imatinib, nilotinib might be effective in some cGvHD patients.

I.5 Rapamycin, a mTor inhibitor

Rapamycin (Sirolimus®), a cyclic macrolid isolated from *Streptomyces hygroscopicus*, was discovered in the 1970's on Easter Islands. This compound has immunosuppressive properties and acts also as an antibiotic and antifungal agent [310-316]. Although this molecule was discovered 40 years ago, its cellular target, the TOR protein, was discovered later in yeast. The mammalian homolog of this protein is termed mammalian Target of Rapamycin (mTor) [317].

I.5.1 mTor, the mammalian Target of Rapamycin

mTor is a serine/threonine kinase that plays a central role in growth, proliferation, metabolism and homeostasis [318-321]. mTor can take part in two different multiprotein complexes: mTORC1 and mTORC2. These two complexes differ by

their scaffolding protein: mTORC1 contains RAPTOR (regulatory-associated protein of mTor) while mTORC2 is linked to RICTOR (rapamycin-insensitive companion of mTor) and MAPKAP1 [322].

Aberrantly elevated activity of mTOR has been observed in human malignancies, and mTOR inhibitors are currently used in the treatment of advanced breast cancer, renal cell carcinoma, and lymphoma [323]. In addition, mTOR signaling also plays an essential role in both innate and adaptative immune responses [324].

I.5.2 Role and activation of mTor

mTOR is regulated by environmental cues in the form of nutrients, growth factors, energy, and stress [325]. Although much is known about how these inputs regulate mTORC1 signaling, there are surprisingly few data connecting mTORC2 to these stimuli.

Thanks to small changes in their molecular compositions, the two complexes regulate different cellular processes in response to different stimuli. Indeed while mTORC1 is responsible for cell cycle progression, cellular growth, macromolecule biosynthesis and autophagy, mTORC2 is important for cell survival, cytoskeletal organization, but the two complexes play a role in metabolism [326].

In addition to its role in metabolism, mTORC1 also plays a role in mitochondrial biosynthesis and autophagy [326]. Indeed, mTORC1 inhibition leads to an increase in autophagy, whereas mitochondrial biosynthesis is enhanced in the setting of increased mTORC1 activation. In the latter case, mTORC1 was shown to promote the transcriptional activity of PPARγ coactivator 1 (PGC1a), a factor that is necessary for mitochondrial biogenesis and oxidative metabolism [325].

mTORC2 controls survival and cell proliferation via the Akt/Protein kinase B pathway. Activity of the mTORC2 complex can be measured by the phosphorylation of the Ser-473 on Akt complexes. Activation of this pathway induces Foxo1 and Foxo3a transcription factor inactivation. These proteins are important for apoptosis and cell cycle regulation. mTORC2 is also implicated in actin reorganization via the PKC-α and RHO proteins [327, 328].

I.5.3 Rapamycin

mTor inhibition by rapamycin is responsible for different consequences in both innate and adaptative immune systems. The inhibitory effects of rapamycin are based on the formation of an allosteric complex with the intracellular FKBP12 protein. The complex inhibits mTORC1 activation by preventing the binding of RAPTOR to the FRB (FKBP12-rapamycin-binding domain) region of mTOR [329]. However, rapamycin treatment abrogates feedback inhibition of mTORC1 on Akt

signaling. This effect associated with weak inhibitory effects on downstream target of mTORC1 such as eIF4-binding proteins lead to an attenuation of its therapeutic effects [330].

Although, mTORC2 does not interact with FKBP12-rapamycin, rapamycin inhibits the assembly of mTORC2 and reduces mTORC2 levels below those needed to maintain Akt/PKB signaling in many cell types [331].

Studies using rapamycin have highlighted its regulatory role on the different immune cell subsets. First, rapamycin exerts its immunosuppressive effects on $CD8^+$ T cells by inhibiting cell cycle progression, proliferation and activation [332, 333]. Recent data have also unraveled that mTor and rapamycin affect trafficking, differentiation, and favor memory $CD8^+$ T-cell generation [334, 335].

mTOR deletion also results in the inhibition of Th1, Th2, Th17 and T_{reg} $CD4^+$ differentiation [336], highlighting its role in Th subset differentiation. The involvement of mTOR in the differentiation of T_{regs} was indicated by the *in vitro* and *in vivo* observations that rapamycin can selectively promote $Foxp3^+$ T cells [337, 338]. Moreover, in the presence of rapamycin, T_{regs} are more resistant to apoptosis. This resistance is possibly mediated by the upregulation of the Pim-2 serine/threonine kinase. Indeed, upon mTOR inhibition, the expression of Pim-2 increases. These results could explain the tolerogenic effect of rapamycin by its ability to preferentially promote T_{reg} differentiation [339-341]. These data also suggest that mTOR has a regulatory role in T_{reg} differentiation. mTor activity is also important for T_{reg} proliferation. Indeed, *in vitro* inhibition of mTOR with rapamycin led to increased proliferation of T_{regs} [337]. However, in repleted wild-type murine models, rapamycin significantly inhibited both homeostatic and alloantigen-induced proliferation of T_{regs}, and promotes their apoptosis, leading to significant T_{reg} depletion. These findings suggest that the effects of rapamycin on T_{reg} survival, homeostasis, and induction, depend heavily on the cellular milieu and degree of activation [342].

Development, survival, function and resting of B cells are also tightly regulated by mTor. However, there is a relative paucity of data regarding the role of mTor in B cells. Splenic B cell proliferation was also shown to be inhibited by rapamycin [343].

Finally, sensing the immune microenvironnement is an important function of mTor. Given that APCs are specialized in the establishment of an efficient adaptive immune response, it is not surprising that mTor plays a role in their differentiation and function. Indeed, mTor influences fast production of type-I interferon by plasmacytoid DCs since plasmacitoid DCs treated with rapamycin are unable to mount a correct response [344, 345]. DC maturation is also tightly regulated by mTor since rapamycin inhibits Flt3 ligand-mediated DC maturation. Finally, DCs matured in the presence of rapamycin can promote T-cell tolerance by inducing

deletion and anergy of effector cells, as well as by promoting the generation of regulatory cells. Indeed, T cells stimulated by rapamycin-matured DCs are anergic. *In vivo*, these rapamycin-derived allogeneic DCs can induce tolerance too, leading to the prevention of graft rejection in mouse models of solid organ transplantation [346-349].

In summary, the antiproliferative capacity of rapamycin on DCs, B cells, $CD8^+$ T cells and T convs and its beneficial effect on T_{regs} make this immunosuppressive agent, alone or in combination with other immunosuppressors, a good therapeutic strategy to consider for allo-HSCT, in both acute and chronic GvHD prevention and treatment and in auto-immune disorders.

	Main effects of rapamycin
$CD8^+$ T cells	Inhibition of cell cycle progression, activation, trafficking and differentiation; Favors memory $CD8^+$ T-cell generation
$CD4^+$ T cells	Inhibition of Th1, Th2, Th17 differentiation
T_{regs}	Favors T_{reg} generation and proliferation; resistance to apoptosis
B cells	Inhibition of proliferation, development, survival and function
DCs	Inhibition of maturation and cytokine production; Induction of T-cell tolerance

Table 4: Most important effects of rapamycin on major immune cell subsets.

I.5.4 Rapamycin for GvHD prophylaxis

As mentioned earlier, standard GvHD prophylaxis is based on a combination of calcineurin inhibitor with MTX. Recently, a combination of tacrolimus with rapamycin was assessed in a number of clinical trials. While initial results appeared promising [350-352], a recent phase III study by the Bone Marrow Transplant Clinical trials Network (BMT-CTN) suggested that combination of a calcineurin inhibitor with either MTX or sirolimus results in similar progression-free survival (PFS), although the incidence of severe aGvHD and of mucositis was reduced in the sirolimus arm [353].

I.5.4.1 Rapamycin as primary treatment for aGvHD

A recent study reported data from 32 patients given rapamycin as first-line treatment for aGvHD [354]. Rapamycin was chosen as first-line treatment, either because of older age (median 60 years), to avoid steroid toxicity, or due to active malignancy at time of transplantation. At time of inclusion, 4 patients (13%) had grade I, 24 (75%) grade II, and 4 (13%) grade III aGvHD. Of the 32 patients, 16 achieved CR. Initial overall response was achieved in 7 days, while median time to CR was 14 days. The remaining patients were refractory to sirolimus alone and received prednisone at a median dose of 0.5 mg/Kg (range, 0.2-1 mg/Kg). Resolution of aGvHD was observed in 12 patients. However, cGvHD developed in 17 patients (55%). The 1-year OS, Relapse-Free Survival (RSF), relapse rate and Non-Relapse Mortality (NRM), were 56%, 37%, 37%, and 20%, respectively. Toxicities included thrombotic microangiopathy, veno-occlusive disease, and CMV reactivation. This study suggests that rapamycin might be an alternative for first-line corticosteroid treatment in grade II-III aGvHD requiring systemic therapy.

More information on rapamycin as aGvHD treatment is available in the context of steroid-refractory aGvHD, for which no specific guidelines for second-line treatments are available. Encouraging data were reported in two retrospective studies (***Table 5***) [355, 356]. In both studies, immunosuppressive treatments (methylprednisone) were maintained at the initiation of rapamycin and a rapamycin-loading dose was first administered followed by daily doses (2 to 4 mg/Kg) to reach a serum level of 7 to 13 ng/mL. OS at 1 year was 44% in the Hoda study with a CR rate of 44%. Definitive control of the disease was 32%. While survival was similar in the Ghez study (42% at 1 year), the authors reported a higher CR rate (86%) and a higher incidence of definitive control of the disease (72%).

	Ghez et al. [355]	*Hoda* et al. [356]
Patients	22	34
Rapamycin dose (Target level)	2 mg/day (5-10ng/mL)	2 mg/day(7-12 ng/mL
Response	91% ORR; 86% CR	76% ORR; 44% CR
OS (median follow-up)	41% (1 year)	44% (1 year)

Table 5: Most important studies evaluating rapamycin for steroid-refractory aGvHD in the clinical setting. CR = Complete response; ORR = Overall Response Rate.

I.5.4.2 Therapy for steroid-refractory cGvHD

Due to its antifibrotic property in addition to its immunosuppressive activities, rapamycin seems to be a promising agent for the treatment of cGvHD. Despite this, only a few studies have assessed rapamycin as a salvage therapy in patients with steroid-refractory cGvHD (***Table 6***) [136, 357]. In the two studies, prior GvHD treatment remained the same at rapamycin initiation and was tapered in case of GvHD control. In *Jurado* the study, OS at 3 years was 57% with an Overal Response Rate (ORR) of 81% and 38% CR. Adverse effects included renal toxicity, TMA, lipidemia and cytopenia. In the study by *Courriel*, OS at 3 years was 63% with 17% CR for cGvHD. The ORR was 63% with a CR rate of 17%. Adverse events reported in this study were the same as in the *Jurado* studies.

	Jurado et al. [357]	*Courriel* et al. [136]
Patients	47	35
Rapamycin dose (Target level)	2 mg/day (5-10 ng/mL)	2 mg/day (7-12 ng/mL)
Response	81% ORR; 38% CR	63% ORR; 17% CR
OS (median follow-up)	57% (3 years)	63% (3 years)

Table 6: Most important studies evaluating rapamycin for steroid-refractory cGvHD in the clinical setting. CR = Complete response; ORR = Overall Response Rate.

I.5.4.3 Therapy for sclerodermatous cGvHD

mTOR inhibition with rapamycin or everolimus (a mTOR-1 specific inhibitor) was assessed in patients with scl-cGvHD patients [358]. The majority of the patients was treated with a mTor inhibitor in combination with other immunosuppresors (MMF, steroids, …). The ORR was 76%, with CR in 17.6% and PR in 58.8% of the patients. Median time to PR for all patients was 3 months. Steroid-sparing effect was observed in 26% of patients who stopped steroids 3 months after treatment initiation. At the end of follow-up, 52% had completely discontinued steroid therapy. Projected OS at 3 years was 72% with no significant difference observed between rapamycin and everolimus.

This study confirms the efficacy of rapamycin in cGvHD treatment, particularly with skin fibrosis. The associated steroid-sparing effect of raparycin treatment represents a major advantage over other immunosuppressors considering the deleterious effect and burden of long-term corticosteroid treatment. Finally, an ongoing randomized phase II/III, multicenter trial is comparing sirolimus plus

prednisone, sirolimus/ECP plus prednisone, and sirolimus/ calcineurin inhibitors plus prednisone for the treatment of cGvHD. The results of this study will help clarify the role and efficacy of sirolimus on specific organ involvement and the optimal combination for the treatment of cGvHD (number BMT CTN 0801).

II. Objectives of the work

Throughout this work, we aimed at better understand mechanisms underlying scl-cGvHD and evaluate therapeutic options, like imatinib and rapamycin, for patients suffering scl-cGvHD following allo-HSCT.

1) *In a first section*, we studied the impact of the TKIs, imatinib and nilotinib, on HSC differentiation and engraftment. Indeed, combining allo-HSCT with TKIs could maximize antileukemic activity against Philadelphia chromosome-positive leukemias. Furthermore, as exposed earlier, imatinib and nilotinib not only inhibit BCR-ABL but also, the receptor for the stem cell factor (SCF), which constitutes an important tyrosine kinase for stem cell biology. However, reconstitution of a fully functional hematopoietic system is crucial for transplantation outcomes and the impact of TKIs on HSCs early after allo-HSCT is unknown. *Pirson* et al. (2006) have previously demonstrated that imatinib inhibits progenitor cell growth in vitro, but does not interfere with engraftment of human HSCs in a xenogenic transplantation model. However, what about nilotinib since little information is available on this molecule and its effect on normal hematopoiesis and HSC engraftment? In a first study, we compared the impact of TKIs, imatinib and nilotinib, on HSC biology both *in vitro* and in a xenogeneic HSC engraftment model in NSG mice. This study has highlighted that (1) both TKIs have no significant side effects on normal hematopoiesis and (2) combining non-myeloablative conditioning regimens with TKIs for Philadelphia chromosome-positive leukemic patients in order to maximize the graft-versus-leukemia effect could be a safe approach with both nilotinib and imatinib.

2) Scl-cGvHD is one of the most severe forms of cGvHD and occurs in up to 15% of these patients. As mentioned earlier, pro-fibrotic pathways, such as the TGF-β (c-Abl pathway) and the PDGF-r pathways, are the major effectors in this inflammatory process. Numerous animal and clinical studies have highlighted the anti-fibrotic potential of imatinib. Indeed, in animal model, imatinib prevents production of fibronectin by blockade of PDGF-β and TGF-β pathways. Blockade of TGF-β fibrogenesis was not only explained by inhibition of the TGF-β-r signaling, but also by c-Abl inhibition. Moreover, imatinib also has immunomodulatory effects on T-, B- and T_{reg} cells *in vitro*. More importantly, clinical studies conducted by *Olivieri* and colleagues have suggested that imatinib was safe and might significantly improve fibrosis. However, two subsequent studies reported limited efficacy of imatinib in 9 cGvHD (*Stadler*, 2009) and 39 steroids-refractory patients (*de Masson*, 2012). The conflicting results from these studies underline the importance to re-assess TKIs for scl-cGvHD in pre-clinical model. *Zerr* et al. (2012) recently assessed the impact of imatinib or nilotinib on cGvHD in a murine scl-cGvHD model (B10.D2→Balb/c). Interestingly, both TKIs prevented experimental cGvHD by blocking aberrant

activation of c-Abl and PDGF-r. However, murine scl-cGvHD was induced with the lowest T cell dose necessary to initiated the pathophysiological processes in that model ($2x10^6$ spleen cells/mice) and, therefore, might not represents the real effect of these TKIs in cGvHD patients with complications or affected by the most severe forms of scl-cGvHD. In this second study, we studied the potential impact of imatinib on fibrosis in the same murine model of scl-cGvHD but with injecting many more spleen cells ($70x10^6$ spleen cells/mice).

3) Finally, in order to still decrease scl-cGvHD and since imatinib failed to prevent this disease and ameliorate survival of recipient mice, we next wanted to study the possibility to increase T_{reg} cell reconstitution following allo-HSCT. Indeed, a correlation between high numbers of T_{regs} in blood and reduced incidence of cGvHD has been found, with activated ($CD103^+$) T_{regs} and low dose administrations of IL-2 showed to increase T_{reg} numbers and improved cGvHD. mTOR inhibitors such as rapamycin are current treatment options for GvHD prophylaxis and for patients with steroid-refractory cGVHD. Rapamycin acts by inhibiting IL-2 signaling in T convs, thereby preventing their proliferation and cell cycle entry, without inhibiting T_{regs}. Indeed, rapamycin enriches for T_{regs} by both favoring *de novo* generation of T_{regs} from naive $CD4^+$ T cells and by selectively expanding T_{regs}. Importantly, it was also recently reported that rapamycin also favors $CD8^+$ T-cell migration into secondary lymphoid tissues by promoting CD62L and CCR7 expression. $CD8^+$ memory immunity is also favored since rapamycin promotes naïve $CD8^+$ T-cell differentiation. Finally, this immunosuppressant also inhibits fibrosis by acting via the PI3K/Akt signaling pathway. Altogether, these data suggest thus that rapamycin could potentially be a good candidate for scl-cGvHD. However, mechanisms of GvHD prevention/improvement by rapamycin are still not fully understood. In this final study, we aimed at better characterizing the effects of rapamycin on scl-cGvHD and to elucidate the biological mechanisms by which it exerts its effects.

III. Imatinib and nilotinib inhibit hematopoietic progenitor cell growth, but do not prevent adhesion, migration and engraftment of human cord blood CD34$^+$ cells

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Background

The availability of tyrosine kinase inhibitors (TKIs) has considerably changed the management of Philadelphia chromosome positive leukemia. The BCR-ABL inhibitor imatinib is also known to inhibit the tyrosine kinase of the stem cell factor receptor, c-Kit. Nilotinib is 30 times more potent than imatinib towards BCR-ABL *in vitro*. Studies in healthy volunteers and patients with chronic myelogenous leukemia or gastrointestinal stromal tumors have shown that therapeutic doses of nilotinib deliver drug levels similar to those of imatinib. The aim of this study was to compare the inhibitory effects of imatinib and nilotinib on proliferation, differentiation, adhesion, migration and engraftment capacities of human cord blood CD34$^+$ cells.

Design and Methods

After a 48-hour cell culture with or without TKIs, CFC, LTC-IC, migration, adhesion and cell cycle analysis were performed. In a second time, the impact of these TKIs on engraftment was assessed in a xenotransplantation model using NOD/SCID/IL-2Rγ (null) mice.

Results

TKIs did not affect LTC-IC frequencies despite *in vitro* inhibition of CFC formation due to inhibition of CD34$^+$ cell cycle entry. Adhesion of CD34$^+$ cells to retronectin was reduced in the presence of either imatinib or nilotinib but only at high concentrations. Migration through a SDF-1α gradient was not changed by cell culture in the presence of TKIs. Finally, bone marrow cellularity and human chimerism were not affected by daily doses of imatinib and nilotinib in a xenogenic transplantation model. No significant difference was seen between TKIs given the equivalent affinity of imatinib and nilotinib for KIT.

Conclusions

These data suggest that combining non-myeloablative conditioning regimen with TKIs starting the day of the transplantation could be safe.

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INTRODUCTION

The Philadelphia (Ph) chromosome results from a reciprocal translocation between chromosomes 9 and 22 resulting in a chimeric *Bcr-Abl* gene. The Ph chromosome translocation is present in 95% and 20-30% of patients with chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL), respectively [1]. BCR-ABL proteins possess a constitutive tyrosine kinase activity and play a key role in signaling pathways resulting in the malignant phenotype of hematopoietic stem cells (HSC).

Imatinib (STI571, imatinib, Glivec®; Novartis Pharmaceuticals) is a competitive inhibitor of ATP for binding to BCR-ABL [2] that induces apoptosis in BCR-ABL dependent cells. As a tyrosine kinase inhibitor (TKI), imatinib is not specific towards BCR-ABL, but also inhibits several other kinases including c-Kit, PDGFR, DDR and Abl [3–5]. Recently, nilotinib (AMN 107, Tasigna®; Novartis Pharmaceuticals) has been developed with the aim of increasing both potency and selectivity towards BCR-ABL [6]. Nilotinib markedly differs from imatinib in its interactions with the BCR-ABL protein [5,6] and is 30 times more potent than imatinib against the BCR-ABL *in vitro* activity in several Ph^+ cell lines [7] and is active against most imatinib-resistant BCR-ABL mutations, but not against the T315I mutant [8,9].

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative treatment for patients with Ph^+ ALL. The majority of older adults with ALL are candidates for a reduced-intensity or a non-myeloablative conditioning regimen [10,11]. Combining allo-HSCT with TKIs could maximize antileukemic activity against Ph chromosome-positive leukemias [12]. In addition to BCR-ABL, imatinib and nilotinib inhibit c-Kit, the receptor for the stem cell factor (SCF). KIT plays an important role in stem cell biology suppressing apoptosis, inducing cell cycle entry [13], promoting colony growth [14], mediating stem cell self-renewal *in vivo* [15], regulating cell adhesion to fibronectin [16] and mediating chemokinetic and chemotactic signals [17].

However, the impacts of imatinib and nilotinib on HSCs during the post-transplantation period are unknown. Reconstitution of a fully functional hematopoietic system is critical for transplantation outcomes. We have previously shown that imatinib inhibits progenitor cell growth *in vitro,* but does not interfere with engraftment of human hematopoietic stem cells in a xenogenic transplantation model [18]. However, very little information on the toxicity of nilotinib on normal hematopoiesis is available and its effects on HSC engraftment are not known. In this study, we have tested, both *in vitro* and *in vivo*, the inhibitory effects of imatinib and nilotinib on proliferation, differentiation and engraftment capacities of human cord blood $CD34^+$ HSCs.

MATERIALS AND METHODS

Isolation of cord blood CD34$^+$ cells

After written informed consent of the mother, cord blood was collected according to the standard procedures of the Cord Blood Bank of the University Hospital of Liège. Mononuclear cells were isolated by centrifugation for 40 minutes at room temperature with Ficoll Paque^{TM} plus density gradient (GE Healthcare, Uppsala, Sweden) and washed twice in phosphate-buffered-saline (PBS) (Lonza, Verviers, Belgium) supplemented with 1% Penicillin/Streptomycin (P/S) (Lonza).

CD34$^+$ hematopoietic stem cells were isolated by magnetic separation according to the manufacturer's instructions (Miltenyi Biotech, Gladbach, Germany). First, cells were incubated during 30 minutes at 4°C with a primary anti-CD34 antibody. Cells were washed with PBS + P/S 1% and incubated for 30 minutes at 4°C with a secondary antibody coupled to magnetic beads. Cells were washed in PBS + P/S 1% and passed twice through a MS column (Miltenyi Biotech). CD34$^+$ cells were collected after elution of unlabeled cells through the column. Cells were counted with Trypan Blue, washed in PBS and frozen in Fetal Bovine Serum (FBS) + Dimethylsulfoxide (DMSO) 10% (Vel, Leuven, Belgium).
The purity of the CD34$^+$ cells was assayed by flow cytometry. A total of 50,000 collected cells were labeled for 30 minutes at 4°C with an allophycoerythrin (APC) conjugated anti-CD34 antibody (BD Biosciences, Erembodegem, Belgium) or with the isotype-matched control (BD Biosciences). Cells were washed twice with PBS + P/S 1% and resuspended in PBS + Formaldehyde 1%. Data acquisition was carried out on a FACSCanto II flow cytometer (BD Biosciences). In all experiments, the percentage of CD34$^+$ cells in the starting cell population was higher than 95%.

Western blot

Human CD34$^+$ cells were thawed in Iscove's MDM (IMDM) (Lonza) supplemented with bovine serum albumin, insulin, transferrin (BIT) 20% (Stem Cell Technologies, Grenoble, France) + P/S 1% and washed with PBS + P/S 1%. Cells were counted with Trypan Blue and resuspended in IMDM + BIT 20% + P/S 1% at a concentration of 10,000,000 cells/mL. A total of 1,000,000 CD34$^+$ cells (100 µL) were seeded in 2.4 mL of IMDM + BIT 20% + P/S 1% supplemented with SCF (100 ng/mL), TPO (50 ng/mL) and FLT-3 (100 ng/mL) (PeproTech, Neuilly-Sur-Seine, France). TKIs were added from a stock solution of 10 mM in DMSO to the medium at a final concentration of 1 or 5 µM. Cells were incubated for 48 hours and then collected and lysed. Total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lysis buffer contained 25 mM Hepes, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM dithiothreitol, phosphatase inhibitors (25 mM β-glycerophosphate, 1 mM

Na3VO4, 1 mM NaF) and complete protease inhibitor mixture (Roche Applied Science, Vilvoorde, Belgium). Polyvinylidene fluoride membrane was incubated with 1:1000 rabbit anti-human phospho-c-Kit (Cell Signaling Technology, Leiden, The Netherlands). The membrane was then incubated with anti-rabbit horseradish peroxidase antibody at 1:2000 (GE Healthcare, Diegem, Belgium). Goat anti-human actin conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany) was used at 1:400. Actin signal was used as an internal standard. Finally, the blot was developed using ECL Western Blot detection system (GE Healthcare).

Colony-forming cell assay

$CD34^+$ cells were thawed in Iscove's MDM (IMDM) (Lonza) supplemented with BIT 20% + P/S 1% and washed with PBS + P/S 1%. Cells were counted with Trypan Blue and resuspended in IMDM + BIT 20 % + P/S 1% at a concentration of 50,000 cells/mL. A total of 5,000 $CD34^+$ cells (100 µL) were seeded in 2.4 mL of MethoCult H4100® (Stem Cell Technologies) supplemented with FBS 30%, EPO 3 U/ml, 2-Mercaptoethanol 0.1 mM (Invitrogen, Merelbeke, Belgium), L-Glutamine 2 mM (Lonza), P/S 1%, SCF (Stem cell factor) 50 ng/mL (PeproTech, Neuilly-Sur-Seine, France) and with conditioned medium of the 5637 cell-line. TKIs were added from a stock solution of 10 mM in DMSO to the medium at a final concentration of 1 or 5 µM and cells were incubated for 14 days at 37°C under a 5% CO_2 atmosphere. Colony forming cells (CFCs) were then counted.

Long term culture-initiating cell assay

Absolute frequencies of LTC-ICs in cell suspensions recovered after a 48-hour cell culture incubation with TKIs at a concentration of 1 or 5 µM, or in control medium, were determined by limiting dilution analysis over MS-5 feeder cells. Briefly, the MS-5 feeder cell line was cultured in RPMI 1640 with 10% FBS. Cells were irradiated at 50 Gy and then plated in 96-well plates at 20,000 cells per well in 100 µL long-term culture (LTC) medium consisting of α-MEM supplemented with 8% horse serum, 8% fetal bovine serum, 0.2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all from Lonza), 0.2 mM inositol (Sigma-Aldrich), 0.1 mM 2-mercaptoethanol. Within a week, thawed $CD34^+$ cells were plated in limiting dilution in another 100 µL of LTC medium and maintained at 33°C in a 100% humidified atmosphere containing 5% CO_2, with weekly half-medium change. After 6 weeks, medium was carefully aspirated from each well, followed by the addition of 200 µL of fully supplemented MethoCult. After an additional 2 weeks, wells were scored for the presence or absence of hematopoietic colonies, and the frequency of LTC-ICs was calculated using L-calc software (Stem Cell Technologies).

Cell cycle analysis

Thawed $CD34^+$ cells were counted with Trypan Blue and resuspended at a concentration of $1x10^6$ cells/mL. A total of $1x10^5$ cells/well (100 µL) was seeded in a 6-well plate, containing 2.4 mL of IMDM supplemented with BIT 20%, P/S 1%, SCF (100 ng/mL), TPO (50 ng/mL) and FLT-3 (100 ng/mL) (PeproTech). TKIs were added from a stock solution of 10 mM in DMSO at final concentrations of 1 µM or 5 µM. Flow cytometric cell cycle analyses of $CD34^+$ cells cultivated during 48 hours with or without TKIs were performed using the CycleTEST™ Plus DNA Reagent Kit (BD Biosciences) as previously reported [18]. The percentage of cells in the different phases of the cell cycle was determined with Modfit software (BD Biosciences) on at least 20,000 acquired events. The percentage of cells in cycle was calculated as follows: percentage = ((S + G_2/M cells)/Total cells) x 100.

VLA-4, VLA-5 and CXCR-4 expression analysis

Thawed $CD34^+$ cells were counted with Trypan Blue and resuspended at a concentration of $1x10^6$ cells/mL. A total of $1x10^5$ cells/well was seeded in a 6-well plate, containing 2.4 mL of IMDM supplemented with BIT 20%, P/S 1%, SCF (100 ng/mL), TPO (50 ng/mL) and with FLT-3 (100 ng/mL). TKIs were added at final concentrations of 1 µM or 5 µM. After 48 hours of culture, human $CD34^+$ cells were washed twice with PBS + FBS 3% + P/S 1% and were then incubated with FITC-conjugated anti-VLA-4 (BD Biosciences) or FITC-conjugated anti-VLA-5 (BD Biosciences) in combination with PE-conjugated anti-CXCR-4 (BD Biosciences) antibodies for 30 minutes at 4°C in the dark. Cells were then washed twice with PBS + FBS 3% and finally resuspended in pure PBS. Data acquisitions (at least 10,000 events) were performed on a FACSCanto II flow cytometer. Integrin density was expressed as the mean channel fluorescence ratio (MCFR) defined as the mean channel fluorescence (MCF) of CXCR-4 or integrin expression divided by MCF of fluorescence of the unstained control.

Migration assay

Migration assays were performed in 6.5 mm diameter 5 µm pore transwells. A total of $1x10^5$ $CD34^+$ cells were plated in 100 µL of IMDM + BIT 20% + P/S 1% + SCF (100 ng/mL) in the upper chamber of the transwell. The bottom compartment was filled with IMDM supplemented with 20% BIT and 100 ng/mL stromal-derived factor-1 alpha (SDF-1α) (PeproTech). After incubation at 37°C during 4 hours, non-migrating and migrating cells were harvested by two standardized washes using PBS + FBS 3% + P/S 1%. Non-migrating and migrating cells were counted by flow cytometry using Trucount Tubes (BD Biosciences) after staining with an APC-conjugated anti-CD34 antibody. The percentage of non-migrating and migrating cells was calculated relative to the total number of harvested cells.

Adhesion assay

Thawed $CD34^+$ cells were counted with Trypan Blue and resuspended at a concentration of $1x10^6$ cells/mL. An aliquot of $1.5x10^5$ cells/well were seeded in a 12-well plate containing 1 mL IMDM supplemented with BIT 20%, P/S 1%, SCF (100 ng/mL), TPO (50 ng/mL) and FLT-3 (100 ng/mL). TKIs were added from a stock solution of 10 mM in DMSO at final concentrations of 1 µM or 5 µM. After 48 hours of culture, human $CD34^+$ cells were washed twice with PBS + P/S 1%.

Adhesion assays were performed in a 12-well plate. Wells were first coated with retronectin (Takara Bio Inc., Shiga, Japan) at a concentration of 9 $\mu g/cm^2$ during two hours at 37°C. Supernatant was aspirated and wells were then incubated with PBS + BSA 1% + P/S 1% for 30 minutes at room temperature. Wells were finally washed twice with PBS + Hepes 2% + P/S 1%.

A total of 150,000 $CD34^+$ cells resuspended in IMDM + BIT 20% + P/S 1% were added in each well and incubated for 90 minutes at 37°C. Supernatants were collected in polypropylene tubes. Adherent cells were detached by using the non-enzymatic cell dissociation buffer (Sigma) and collected in new polypropylene tubes. Cells were finally stained with an APC-conjugated anti-CD34 antibody and counted by flow cytometry using Trucount Tubes. The percentage of adherent cells was calculated relative to the total number of harvested cells.

Transplantation into NOD/SCID/IL2rγ (null) mice

Six hours before $CD34^+$ cell injection, NOD/SCID/IL-2Rγ (null) (NSG) mice (The Jackson laboratory, Bar Harbor, USA) were irradiated with 2.5 Gy TBI using a ^{137}Cs source. Human $CD34^+$ cells were thawed in IMDM + FBS 10% + P/S 1% and washed in PBS + P/S 1%. Cells were counted with Trypan Blue and resuspended in PBS at a concentration of $3x10^6$ cells/mL ($6x10^5$ cells/200 µL). Mice were inoculated intravenously with $6x10^5$ $CD34^+$ cells. Gavage with TKIs or a placebo was started at day 0. Imatinib was dissolved in sterile water and administrated at a dose of 150 mg/kg/day (50 mg/kg every morning and 100 mg/kg every evening) while nilotinib was prepared in 0.5% hydroxypropylmethyl cellulose (HPMC, Sigma) aqueous solution containing 0.05% Tween 80 and given at a concentration of 75 mg/kg/day (37.5 mg/kg every morning and evening). After 42 days, mice were sacrificed. Bone marrow cells from the two femurs were collected in sterile RPMI + FBS 10% + P/S 1%. Cells were counted with an HORIBA ABX® automatic cell counter (ABX Hematology, Montpellier, France). Cells were stained with anti-human CD45 (BD Biosciences) and anti-mouse CD45 (BD Biosciences) antibodies in order to determine the percentage of human chimerism by FACS analysis. Data acquisition was performed on a FACSCanto II flow cytometer on at least 20,000 mononuclear cells.

Ethics statement

All experiments using NSG mice were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Liège (Permit Number: 712). Mice were maintained in top-filtered cages in a standard animal facility and provided sterilized food and water *ad libitum*. Sterilized water supplemented with Baytril® 1% (Bayer HealthCare, Diegem, Belgium) was given from 3 days before to the end of the experiment. Water was change every 2-3 days. All euthanasia were performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Statistical analyses

Statistical analyses were performed with the GraphPad® Prism 5.00 Software. The paired Student's T test was used to assess the impact of TKI on human cord blood $CD34^+$ cells *in vitro*. Percentages and numbers of human cells in NSG mice were compared with the unpaired Student's T test.

RESULTS

Imatinib and nilotinib inhibited c-Kit receptor phosphorylation in a dose-dependent manner

We have first determined the inhibitory effect of TKIs on the c-Kit receptor cell signaling by western blot (n = 4). Human $CD34^+$ cord blood HSCs were cultured in cytokine-supplemented medium for 48 hours with or without TKIs, washed and then lysed. Proteins were extracted on ice and dosed for western blotting analysis. The c-Kit phosphorylation levels in human $CD34^+$ cells were decreased in a dose-dependent manner. Indeed at the highest concentration, both imatinib and nilotinib decreased dramatically the band intensity of phospho-c-Kit. No differences were seen between imatinib and nilotinib at a concentration of 1 µM and 5 µM (***Figure 1***).

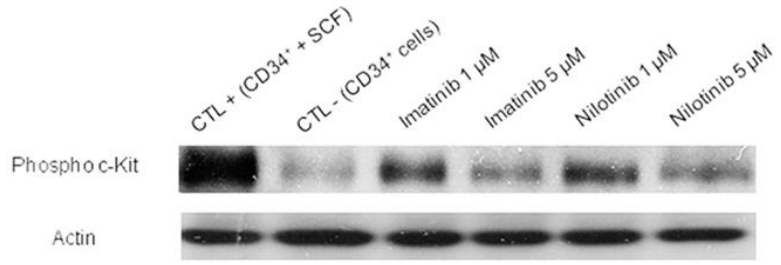

Figure 1. Inhibitory effects of TKIs on c-Kit phosphorylation in human $CD34^+$ cord blood HSCs. The phosphorylated c-Kit receptor was detected by western blot after a 48-hour culture in presence of either imatinib or nilotinib. Representative picture from 4 independent experiments (n = 4).

Both imatinib and nilotinib inhibited formation of precursor colony-forming cells

To determine whether imatinib and nilotinib could inhibit the generation of hematopoietic precursors, colony-forming cell (CFC) assays were carried out. In a first set of experiments (n = 4), $CD34^+$ cells were cultured for 48 hours with or without TKIs, washed and then plated in cytokine-supplemented MethoCult for 14 days. Both imatinib and nilotinib significantly inhibited CFC formation. Indeed, imatinib, at a concentration of 1 or 5 µM, decreased CFC formation by a mean ± SD of 24.91 ± 14.05% (p = 0.0415) and 49.66 ± 30.19% (p = 0.0461) respectively, while nilotinib, at the same concentrations, reduced CFC numbers by 25.15 ± 12.36% (p = 0.0268 and p = 0.8173 in comparison to imatinib) and 54.81 ± 34.39% (p = 0.0498 and p = 0.1314 in comparison to imatinib), respectively (***Figure 2A***).

Next, cord blood $CD34^+$ cells were seeded in MethoCult supplemented with or without imatinib or nilotinib at a final concentration of 1 or 5 µM for 14 days. As observed in the first series of experiments, TKIs significantly diminished CFC generation. Imatinib reduced colony formation by 41.67 ± 14% (p = 0.0356) at a concentration of 1 µM and 79.42 ± 9.309% (p = 0.0045) at 5 µM. Nilotinib decreased CFC formation by 19.64 ± 1.90% (p = 0.0031 and p = 0.0898 in comparison to imatinib) and 78.28 ± 5.27% (p = 0.0015 and p = 0.6733 in comparison to imatinib), respectively (n = 3, ***Figure 2B***).

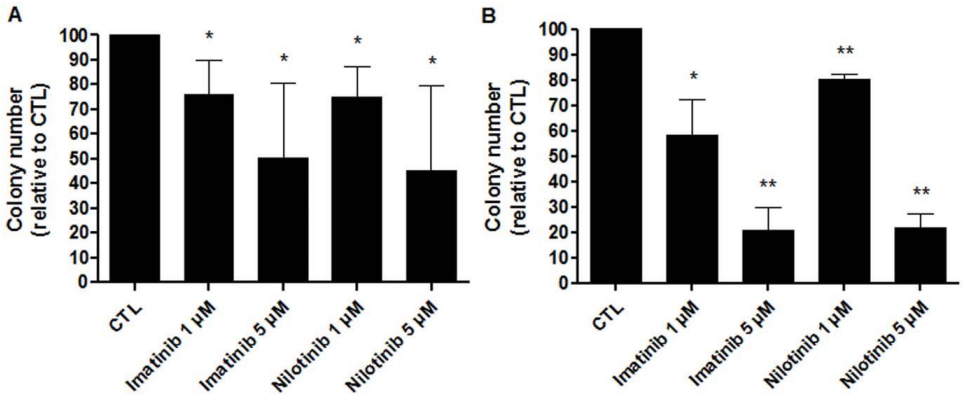

Figure 2. TKIs dramatically decrease CFC formation. (A): Influence of a 48-hour pre-culture in the presence of either imatinib or nilotinib on CFC formation. **(B):** CFC generation in CFC assays supplemented with TKIs. Results are expressed as mean percentages relative to control experiments without TKIs ± SD. n = 4, *p<0.05, **p<0.005 versus CTL, Student's paired t tests. CTL: control condition without TKIs

Imatinib and nilotinib did not decrease absolute frequencies of LTC-ICs

The capacity of TKIs to inhibit the differentiation of primitive hematopoietic progenitors was first assessed in long-term cultures. In a first set of experiments (n = 3), human cord blood $CD34^+$ cells were incubated for 48 hours in the presence/absence of imatinib or nilotinib at a final concentration of 1 and 5 µM and then seeded in 96-well plates for LTC-IC assays without any TKIs. No significant differences were seen in the absolute frequencies of LTC-IC in each condition (***Figure 3A***).

In a second set of experiments (n = 3), $CD34^+$ cells were directly plated for LTC-IC assays and incubated in the presence/absence of TKIs at a final concentration of 1 or 5 µM. Inhibitors were also added at all weekly half-medium change. As observed above, neither imatinib nor nilotinib decreased absolute frequencies of LTC-ICs (***Figure 3B***).

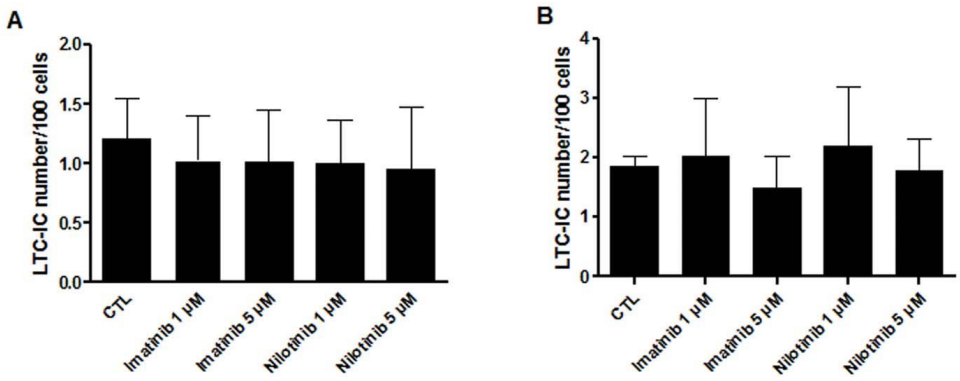

Figure 3. Neither imatinib nor nilotinib induce a decrease of LTC-IC frequency. **(A):** Influence of a 48-hour pre-culture with imatinib or nilotinib at a final concentration of 1 or 5 µM on LTC-IC frequencies. **(B):** LTC-IC generation with TKIs in a 6-week LTC-IC assay. Results are expressed as mean ± SD. n = 3, Student's paired t tests. CTL: control condition without TKIs

Entry into cell cycle of human cord blood $CD34^+$ cells was impaired in the presence of imatinib and nilotinib

We assessed the impact of a 48-hour culture in the presence of imatinib or nilotinib on cord blood $CD34^+$ cell proliferation. HSC proliferation was markedly reduced in the presence of imatinib 1 µM (73.2 ± 4.5%; n = 3; p = 0.003) or nilotinib 1 µM (68.4 ± 11.4%; n = 3; p = 0.026 and p = 0.5620 in comparison to imatinib), and even more with the presence of 5 µM of imatinib (p = 0.005) or nilotinib (p = 0.008 and p = 0.6746 in comparison to imatinib): HSC proliferation was decreased by 78.25 ± 9.624% and 63.49 ± 12.40% respectively (n = 3) (***Figure 4; p.63***).

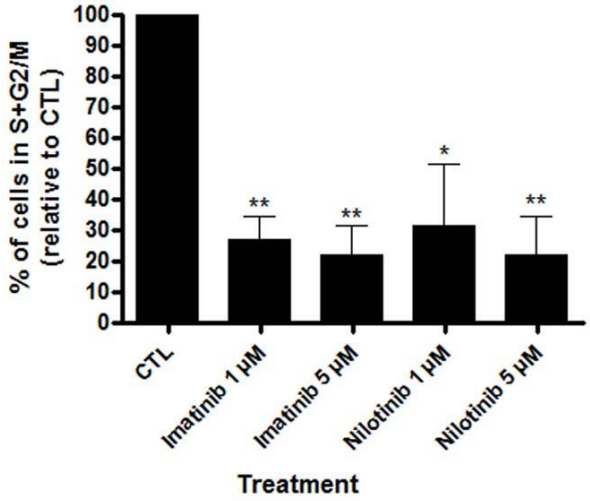

Figure 4. Colony formation in presence of tyrosine kinase inhibitors were decreased due to the inhibition of cell cycle entry. Human cord blood $CD34^+$ were cultured for 48 hours in presence/absence of either imatinib or nilotinib and then stained with propidium iodide using the CycleTEST™ Plus DNA Reagent Kit. Results are expressed as mean percentages relative to control experiments without TKIs ± SD. n = 3, *p<0.05, **p<0.01 versus CTL, Student's paired t tests. CTL: control condition without TKIs

Expression of VLA-4 and VLA-5, but not the CXCR-4, cell surface receptors were decreased after a 48h cell-culture with imatinib and nilotinib

Homing of hematopoietic stem cells is a critical step for the success of allo-HSCT. In this process, three key-players have been identified: VLA-4, VLA-5 and CXCR-4 [19,20]. Mean channel fluorescence ratio (MCFR) of the expression of these receptors on the cell surface of human cord blood $CD34^+$ cells was determined by flow cytometry after a cell culture containing TKIs (or not). Imatinib significantly decreased expression of VLA-4 by a mean ± SD of 10.457 ± 3.058 (p = 0.0032) and 12.520 ± 1.872 (p < 0.0001) at a concentration of 1 and 5 µM, respectively. Nilotinib at the same concentration induced the same effect by decreasing MCFR values by a mean ± SD of 8.920 ± 3.472 (p = 0.0088) and 11.247 ± 2.336 (p = 0.0008), respectively (n = 3) (***Figure 5A; p.64***).

VLA-5 expression was also decreased in the presence of both imatinib and nilotinib. Indeed, MCFR values were decreased by a mean ± SD of 1.307 ± 0.5103 (p = 0.0318) and 1.724 ± 0.4219 (p = 0.0121) for imatinib at 1 and 5 µM, respectively. Nilotinib decreased VLA-5 MCFR values by a mean ± SD of 1.724 ± 0.6833 (p = 0.0733) and 1.424 ± 0.3931 (p = 0.0135) at the same concentration respectively (n = 3) (***Figure 5B; p.64***).

While both VLA-4 and VLA-5 expression were significantly decreased by TKIs, CXCR-4 cell surface expression was not affected upon 48-hour cell culture in the presence of imatinib or nilotinib (n = 6) (***Figure 5C***).

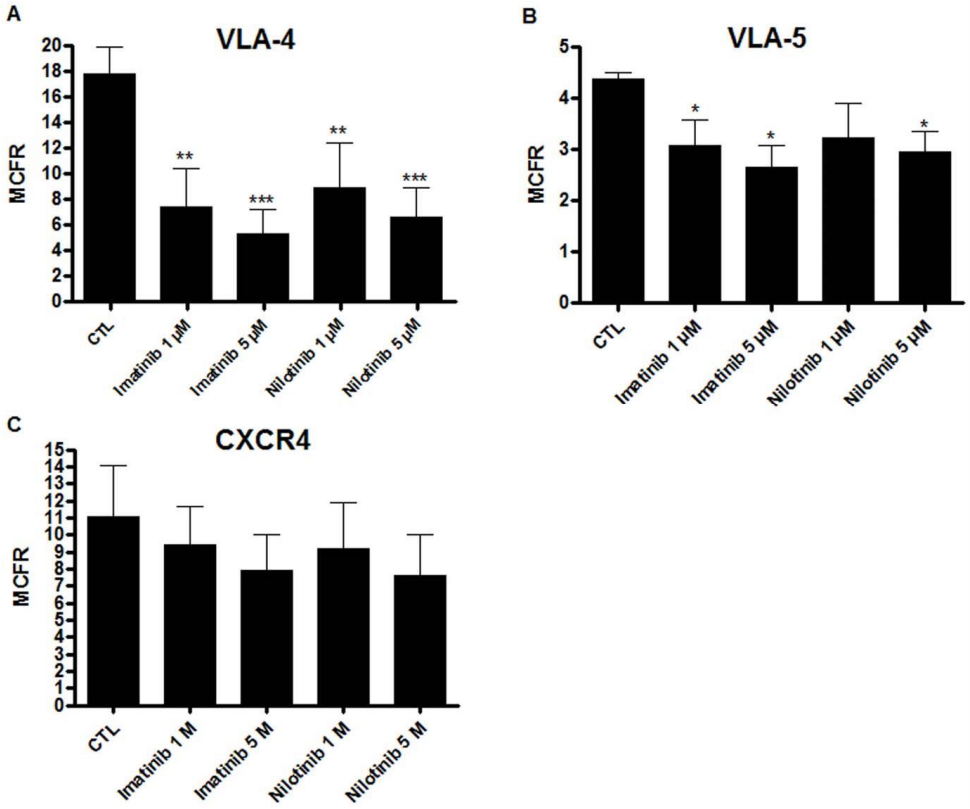

Figure 5. Cell surface expression of VLA-4 and VLA-5 were decreased after a 48h cell-culture with TKIs while CXCR-4 expression was not affected. Influence of 48-hour pre-culture in presence of either imatinib or nilotinib on **(A):** VLA-4 **(B):** VLA-5 and **(C):** CXCR-4. Results are expressed as the mean channel fluorescence ratio (MCFR) ± SD. n ≥ 3, *p<0.05, **p<0.01, ***p<0.001 versus CTL, Student's paired t tests. CTL: control condition without TKIs

Adhesion to retronectin of cord blood CD34$^+$ cells was not modified by 1 µM imatinib and nilotinib

As we observed a decreased in the expression of VLA-4 and VLA-5 in the presence of TKIs, we asked the question whether this lower expression affected the function of these receptors. We thus performed adhesion assays to retronectin with CD34$^+$ cells cultured for 48 hours with TKIs. Imatinib 1 µM did not alter adhesion to retronectin (p = 0.6186) while imatinib 5 µM decreased it by 5.69 ± 4.134% (p = 0.0135) (n = 10). Similarly, nilotinib had no significant

effect on adhesion at 1 µM, while at 5 µM it significantly reduced adhesion by 3.51 ± 5.933% was observed (p = 0.0432) (n = 10) (***Figure 6A***).

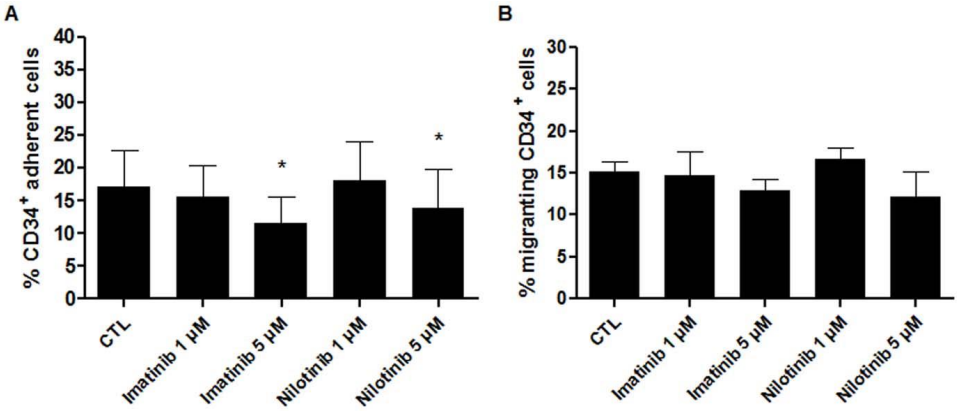

Figure 6. Influence of TKIs on adhesion and migration of human cord blood CD34$^+$ cells. (A): Adhesion of CD34$^+$ cells to retronectin was significantly reduced in presence of the highest doses of TKIs. Results are expressed as the percentage of adherent cells ± SD. n = 10, *p<0.05 versus CTL, Student's paired t tests. **(B):** Migration of human HSCs toward a SDF-1α gradient was not modified by a 48 hour pre-culture period in the presence of TKIs. Results are expressed as the percentage of migrating cells ± SD. n = 4, Student's paired t tests. CTL: control condition without TKIs

A 48-hour incubation with TKIs did not affect the capacity of human CD34$^+$ cells to migrate through a SDF-1α gradient

No significant changes were observed in CXCR-4 expression in the presence of TKIs. To investigate the functional impact of these observations, migration assays of CD34$^+$ HSCs were performed. CD34$^+$ cells cultivated for 48 hours in the presence of TKIs were seeded in the upper chamber of a transwell containing SCF-supplemented medium. The lower chamber was filled with medium containing SDF-1α. Migration through the filter was allowed during 4 hours at 37°C. No significant difference were observed between cells cultivated with TKIs in comparison to the control (n = 4) (***Figure 6B***).

Daily dosing of either imatinib or nilotinib did not affect repopulating activity in NSG mice

Twenty-five sublethally irradiated NSG mice (in 3 independent experiments) were injected intravenously with 6x10^5 human CD34$^+$ cells and treated orally with placebo, imatinib 150 mg/kg/day or nilotinib 75 mg/kg/day for 42 days starting on day 0. No death occurred before the end of the experiments. Bone marrow cellularity was similar in the three groups. Numbers of cells/femur were (expressed as mean ± SD) 5.66 ± 4.14 x10^6 in control mice, 3.55 ± 1.67 x10^6 in mice treated with imatinib (p = 0.1982) and 4.17 ± 2.74 x10^6 in nilotinib-

treated mice (p = 0.3731) (***Figure 7A***). Bone marrow chimerism was analyzed by flow cytometry. No significant differences were seen between mice treated placebo (52.5 ± 2.7%; n = 9) or with imatinib (47.7 ± 5.3%; n = 8; p = 0.4130), while engraftment of human $CD34^+$ cells was slightly decreased (40.6 ± 4.4%; n = 8; p = 0.0314) in mice treated with nilotinib (***Figure 7B***).

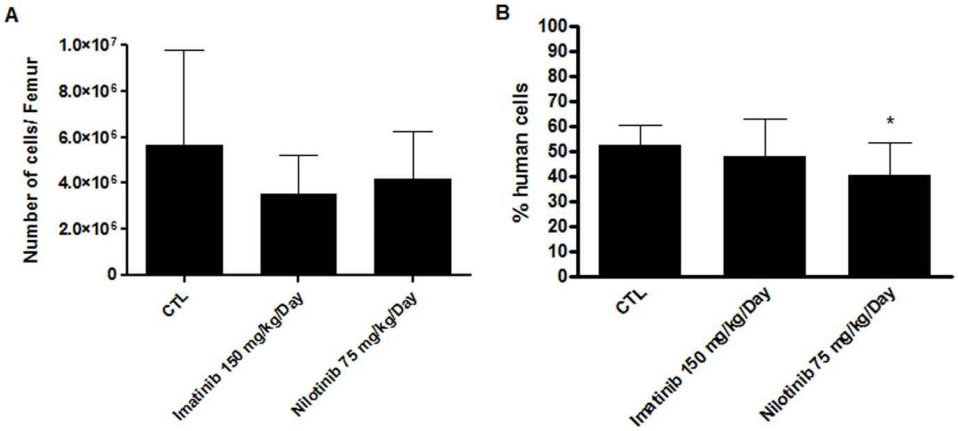

Figure 7. Effects of TKIs in a mouse model of transplantation. (A): Daily doses of TKIs for 42 days did not affect bone marrow cellularity. Results are expressed as the mean number of cells per femur ± SD. n ≥ 8, Student's unpaired t tests. **(B):** Effect of continuous administration of TKIs on bone marrow chimerism. Imatinib did not affect the percentage of human cells in the bone marrow while nilotinib slightly decreased it compared to the control group. Results are expressed as the mean percentage ± SD. n ≥ 8, *p = 0.0314 versus CTL, Student's unpaired t tests. CTL: control condition without TKIs

DISCUSSION

The aim of this project was to assess the impact of TKIs on hematopoietic stem cell function engraftment. Retrospective studies have suggested that treatment with TKIs before allo-HSCT did not preclude the outcome of engraftment and did not increase transplant-related toxicity [21–23]. Prospective studies showed that early administration of TKIs after allo-HSCT at a dose intensity comparable to that used in primary therapy seem to be safe [24] and can result in favorable long-term survival [12]. But the vast majority of these patients did not take TKIs during the engraftment period to prevent graft delay or failure. However, in the setting of non-myeloablative allo-HSCT for blast-crisis CML or Ph^+ ALL, a 2-4 week TKI discontinuation may expose patients to early relapses. These data prompted us to evaluate the inhibitory effects of imatinib and nilotinib on the proliferation, differentiation and engraftment capacities of human cord blood $CD34^+$ HSC.

The effects of TKIs were first assayed on LTC-ICs by limiting dilutions. Our data show that neither imatinib nor nilotinib had a significant impact on primitive progenitors. Indeed, as described before, LTC-IC frequencies were not affected by a 48 hour pre-culture or a 6-week incubation with TKIs [25], confirming previous findings that primitive HSC are less sensitive to TKIs than committed progenitors, possibly related to an enhanced presence of efflux drugs transporters in primitive HSCs [26].

We also evaluated the effects of TKIs on CFCs. Unlike LTC-IC frequencies, CFC growth was significantly decreased in culture containing either imatinib or nilotinib. Moreover, their inhibitory effects were permanent since a 48-hour pre-culture with TKIs is sufficient to decrease significantly the CFC generation definitively. Two hypotheses could explain these observations: (a) increased apoptosis or (b) inhibition of cell cycle entry. Since we and others have previously showed that imatinib do not increase the apoptosis of committed progenitors [18,27], we investigated the proliferation of human $CD34^+$ cells in the presence of TKIs. Our data demonstrate a significant decrease in HSC proliferation. These observations could be explain by the inhibition of others tyrosine kinases by imatinib and nilotinib. Indeed, *Bartolovic et al.* have shown that imatinib exerts growth inhibitory effect on normal $CD34^+$ cells by the inhibition of SCF/c-kit pathway [27]. Moreover, studies on the effects of nilotinib on bone cells in Ph^+ patients receiving nilotinib for treatment of CML have demonstrated that nilotinib potently inhibited osteoblast proliferation through inhibition of the platelet-derived growth factor (PDGFR). Furthermore, inhibition of c-Abl could contribute to the growth inhibition of CFCs by TKIs since antisense strategies have demonstrated that inhibition of c-Abl leads to the accumulation of $CD34^+$ cells in G_0/G_1 and to inhibition of CFU-GM formation [28,29]. Our results confirm also the results of Jorgensen and colleagues which showed that the predominant effect of imatinib and nilotinib on $CD34^+$ CML cells is anti-proliferative rather than pro apoptotic. Indeed, the anti-proliferative effect of TKIs on Ph^+ $CD34^+$ cells is mainly caused by the inhibition of BCR-ABL [30].

Because VLA-4, VLA-5, and CXCR-4 play a major role in the homing of HSCs, we investigated the effect of TKIs on the expression of these surface receptors by flow cytometry. Despite our previous findings that the expression of VLA-4, VLA-5, and CXCR-4 of $CD133^+$ cells was not modified by imatinib [18], a significant decrease in the expression of VLA-4 and VLA-5 was observed with either imatinib or nilotinib. However, no significant differences in CXCR-4 expression on $CD34^+$ cells were seen. These apparent discrepancies could be explained by the cell source since in our previous publication [18], $CD133^+$ cells isolated from peripheral blood of mobilized healthy volunteers were investigated while, in this study, $CD34^+$ cells from cord blood were used in all

experiments. Indeed, despite a higher VLA-4 and VLA-5 expression, cord blood $CD34^+$ cells exhibit a lower CXCR-4 cell surface expression and a higher capacity to regenerate LTC-IC per competitive repopulating unit (CRU) [31] than on peripheral blood HSC cell surface [32,33]. These differences in homing-related molecule expression could explain our discrepancies in the adhesion and migration behavior of cord blood $CD34^+$. We then tested whether the decreased expression of VLA-4 and VLA-5 affected the capacity of $CD34^+$ cells to adhere to retronectin *in vitro*. Adhesion was not affected by imatinib or nilotinib at physiological concentrations (1 µM) but decreased at higher doses (5 µM). $CD34^+$ cell migration towards a SDF-1α gradient was not affected by TKIs. Inverse relationships between migration and adhesion capacities have often been observed. In HSCs, higher cell cycle activity is related with stronger adherence and decreased motility [34,35]. However, because TKIs inhibit $CD34^+$ cell proliferation, their effect on hematopoietic cell adhesion and migration appears to be independent of cell cycle activity. Additional studies will be necessary to investigate the impact of imatinib or nilotinib on tyrosine kinases implicated in adhesion and migration, such as the focal adhesion kinase or the related kinase PYK2 that is expressed in $CD34^+$ cells [36].

Finally, we assessed the impact of TKIs on engraftment in a xenotransplantation model. Numbers of cells in the bone marrow of the femurs were similar in mice treated with placebo, imatinib or nilotinib. Moreover, no significant differences were seen in the percentages of bone marrow human $CD45^+$ cells between mice treated with imatinib or placebo. However, the engraftment of human HSCs was slightly decreased in mice treated with nilotinib. This might be explained by the high daily dose of nilotinib (75 mg/kg/day) used in these experiments. Our results are comparable to those of our previous study [18] and with those of Hoepfl and colleagues, who demonstrated that imatinib (25 mg/kg twice daily) has no significant influence on hematopoietic engraftment in a syngeneic mouse bone marrow transplantation model [37].

On the basis of our data, combining non-myeloablative conditioning with TKIs for Ph^+ ALL patients in order to maximize the graft-versus-leukemia effect could be possible with both nilotinib and imatinib.

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