PDGF-r and TGF-β signaling. Phosphorylation levels of PDGF-r were significantly decreased in imatinib-treated recipients in comparison to control mice, while a similar trend was observed for c-Abl. This is in concordance with the observations reported by Zerr *et al.* in a similar murine model of scl-cGvHD (with the exception that smaller numbers of spleen cells were used in their study) [298].

However, imatinib failed to ameliorate scl-cGvHD in our study. This is in contrast to what has been previously reported by Zerr *et al.* who observed that imatinib significantly decreased scl-cGvHD in a similar scl-cGvHD murine model [298]. Smaller numbers of spleen cells were injected to recipient mice in that study (35 times less; $2x10^6$ versus $70x10^6$), resulting in a less severe scl-cGvHD. These observations could suggest that imatinib when used as a single drug might be mainly efficient in patients with moderate intensity cGvHD. These observations are also in concordance with a recent paper by de *Masson et al.*, in which they observed a limited efficacy of imatinib for patients affected by severe scl-cGvHD.

Imatinib also exerts some inhibitory effects on T-cell proliferation and activation (LCK, c-Kit and c-Abl cell signaling inhibition) *in vitro*. These observations could be explained by cell cycle arrest in phase G_1/G_0, resulting in DNA synthesis inhibition both *in vitro* and *in vivo*. Furthermore, its effect is also dose-dependent and does not induce apoptosis [279, 280]. IL-2, TNF-α and IFN-γ production by T cells are also affected [280, 282]. However, despite these *in vitro* data, we observed a limited impact of imatinib on immune reconstitution following allo-HSCT in this murine model.

The lack of significant benefit from imatinib in the current study contrasts with recent observations from our group in the same mouse model where T-cell modulation with either the mTor inhibitor rapamycin or the demethylating agent azacitidine (Fransollet *et al.*, submitted) were able to significantly ameliorate scl-cGvHD.

As mentioned in section I.3.2.2.2, correlations have been found between high numbers of T_{regs} in blood and a reduced incidence of cGvHD [179, 180] while infusion of donor splenic $CD103^+$ T_{regs} prevented cGvHD in experimental models [183]. However, the role of TKIs in T_{reg} regulation is still incompletely understood. A study has observed impaired immunosuppressive functions and lower FoxP3 expression in T_{regs} cultured with imatinib [283]. However, our data demonstrate that T_{reg} proliferation is not significantly affected by imatinib treatment *in vivo*. T_{reg} immunosuppressive capacity was not assessed in our study and must be addressed in future experiments.

Future studies should assess the combination of imatinib with T-cell modulating agents. Moreover, nilotinib has a better safety profile than imatinib and an equivalent PDGF-r inhibitory profile. The *Oliveiri* group started a phase I-II study, which assessed safety and efficacy of nilotinib in patients with refractory cGvHD [309]. Preliminary data suggest that, like imatinib, nilotinib might be effective in some cGvHD patients.

A prospective phase III clinical study should ideally be conducted to determine the impact of imatinib/nilotinib in scl-cGvHD. This phase III study should also investigate the impact of imatinib/nilotinib on immune recovery.

VI.3 Section III: Rapamycin for scl-cGvHD

Given that imatinib failed to ameliorate scl-cGvHD despite blocking the PDGF-r and TGF-β signaling pathways, we decided to assess the impact of rapamycin as a potential alternative to imatinib. Indeed, mTOR inhibitors such as rapamycin (sirolimus) or everolimus are current treatment options for GvHD prophylaxis and for patients with steroid-refractory cGvHD. However, mechanisms of GvHD prevention/improvement by rapamycin are still not fully understood. Rapamycin acts in part by inhibiting IL-2 signaling in Tconvs (but not in T_{regs} that depend primarily on the STAT5 pathway for IL-2 signaling), thereby inhibiting their proliferation and cell cycle entry. Furthermore, rapamycin also inhibits $CD4^+$ helper T-cell differentiation into Th1 and Th17 cells. Expression of CD62L and CCR7 (favoring their migration into secondary lymphoid tissues) are also promoted by rapamycin, regulating $CD8^+$ T-cell trafficking into lymphoid organs. Finally, generation of memory $CD8^+$ T-cell immunity is also increased with rapamycin treatment. Importantly, rapamycin has been demonstrated to enrich for T_{reg} by both favoring *de novo* generation of T_{reg} from naive $CD4^+$ T cells and by selectively expanding T_{reg}.

Furthermore, rapamycin appears to have antifibrotic properties. Indeed, other experimental studies have demonstrated that rapamycin was also able to prevent *in vivo* fibrogenesis by reducing the levels of inflammatory mediators and preventing the deposition of extracellular matrix, mainly by inhibiting the PI3K/Akt cell signaling pathway. Rapamycin exerts these effects by (1) reducing collagen production by TGF-β-activated fibroblasts; (2) inhibiting the expression of TGF-β1, MMP-2, MMP-9, and TIMP-1; and (3) by directly down-regulating type I and type III collagen synthesis and by up-regulating MMP-1 synthesis.

However, despite these findings, a few studies have assessed mTOR inhibition with rapamycin and/or everolimus (a mTOR-1 specific inhibitor) for scl-cGvHD patients [136, 358]. The majority of the patients was treated with a mTor inhibitor in combination with other immunosuppressors (MMF, steroids, …).

Important results included: (1) The ORR was 76%, with CR and PR in 17.6% and 58.8%, respectively; (2) Median time to PR for all patients was 3 months. At the end of follow-up, 52% had completely discontinued steroid therapy; (3) Projected OS at 3 years was 72% with no significant difference observed between rapamycin and everolimus.

In this final study, as there is a need for more pre-clinical and clinical studies, we assessed the impact of rapamycin in a well-established experimental scl-cGvHD murine model with the aim of dissecting its mechanisms of action.

In accordance with previous reports in murine models, we first showed that experimental cGvHD was significantly ameliorated with rapamycin. Further, we dissected several mechanisms by which rapamycin prevented cGvHD. As expected, *in vivo* T-cell proliferation was decreased by rapamycin treatment. This is in concordance with prior observations showing decreased T-cell proliferation by rapamycin treatment [395, 396]. Several complex mechanisms could mediate this effect: (1) inhibition of IL-2 signaling in T convs, (2) induction of non-protein coding RNA GAS5 and (3) deletion of autophagy-related genes.

Interestingly, contrasting with the observed lower T-cell proliferation in rapamycin-treated mice, we observed higher T-cell numbers in the peripheral blood of rapamycin-treated mice. This could be possibly explained by:

(1) Decreased apoptosis. Indeed, we observed higher levels of BCL-2 expression by both $CD4^+$ and $CD8^+$ T cells in rapamycin-treated than in control mice. These observations are in accordance with previous studies that demonstrated that rapamycin increased the cellular concentration of BCL-2 and had anti-apoptotic effect in a B-cell lymphoma cell line and decreased T-cell apoptosis through autophagy [391, 397].

(2) Decreased T-cell homing into non-lymphoid organs. Indeed, lower T-cell infiltration in the lungs and the liver was observed. This could be explained by lower expression levels of CCR3, CCR5, CXCR3 and CXCR4 by T cells from spleens of rapamycin-treated mice compared to the control group on D+21 post-transplant. Prior studies have already demonstrated that rapamycin reduced CCR5 expression by $CD4^+$ T cells and CXCR4 expression by gastric carcinoma cells. Finally, a higher proportion of CD62L-expressing T cells was found in the rapamycin-treated group, which confirms previous observations showing that rapamycin increases the expression of KLF2 which in turn prevent the downregulation of CD62L and CCR7, thus improving recirculation of T cells into secondary lymphoid tissues.

(3) T-cell differentiation inhibition. Indeed, previous studies have highlighted a prevention of T-cell differentiation by rapamycin. Our study is in line with these observations since we observedhigher frequencies of naive T cells in the rapamycin group. Accordingly, frequencies of effector memory T cells were dramatically decreased by rapamycin.

Finally rapamycin had a mixed impact on T_{regs}. Indeed, rapamycin increased their frequency in the lungs but decreased their frequency in the peripheral blood. However, T_{regs} from the rapamycin-treated mice had higher KI67 and pSTAT5 expression than control mice, suggesting better T_{reg} homeostasis. This suggests that rapamycin effects on T_{regs} may be organ- and model-dependent. This could be supported by a study showing that rapamycin did not increase (and even reduced!) T_{reg} frequencies in lympho-repleted mice. Higher expression of CD103 by T_{regs} from Rapamycin-treated mice was also observed in our study, suggesting that T_{regs} from mice receiving rapamycin might be more efficient at preventing cGvHD.

From this study, we can conclude that rapamycin prevents experimental scl-cGvHD by different mechanisms including decreased T convs proliferation, decreased T-cell differentiation, and decreased T-cell migration towards certain target organs and increased proportions of activated T_{regs}.

VI.4 Perspectives

Even if the previous study suggests that rapamycin could be a good candidate for scl-cGvHD treatment, we must carefully confirm these results in different models of cGvHD. Indeed, as exposed earlier, several models of cGvHD exist but none of these models encompass all features of the human disease. To that end, in a first study, we propose to use rapamycin in the sle-cGvHD and bronchiolitis obliterans murine models.

In light of the encouraging results obtained with the mTor inhibitor rapamycin on scl-cGvHD, we propose to investigate its effect on immune reconstitution. Indeed, as exposed earlier, reconstitution of a fully functional immune system after allo-HSCT is crucial. Patients with cGvHD experience slower immune reconstitution than patient without GvHD. T-cell immunity is particularly important following allo-HSCT and recovery of a functional thymopoiesis is crucial. To that end, we propose to study the impact of this compound on thymic function following allo-HSCT. These studies can be performed both in aGvHD and cGvHD murine models (like the thymus-dependant cGvHD model) since rapamycine is also broadly used in aGvHD prophylaxis.

In 2010, *Esposito et al.* investigated the effect of rapamycin administration in a relapsing-remitting experimental autoimmune encephalomyelitis murine model (EAE). They observed that rapamycin dramatically ameliorated the clinical course EAE, while treatment discontinuation resulted in early reappearance of disease. Reduced central nervous system damages, such as demyelination and axonal loss, were also observed. Rapamycin-mediated mechanisms included: (1) decreased IFN-γ, and IL-17 production by antigen-specific T cells and (2) disappearance of $CD4^+$ effector T cells and selective expansion of $CD103^+$ T_{regs} [412]).

Although damage to gastrointestinal epithelium caused by alloreactive T cells is the primary contributor to aGvHD-related mortality, recent experimental evidence reports that GvHD can be a risk factor for neurological complications (***NC***). NC such as central pareses, seizures, headaches, mental changes, consciousness disturbances, cerebellar signs, and cognitive deficits consistent with encephalopathy, meningoencephalitis, angiitis, atrophy, and demyelination have all been reported in patients who had received an allo-HSCT, and particularly in patients with cGvHD. Historically, NC associated with allo-HSCT have been attributed to the underlying primary disease, drug and radiation toxicities, or infection. These confounding factors have made a comprehensive assessment of the contribution of GvHD to NC in patients receiving allo-HCT technically challenging.

Moreover, psychological distress is associated with NC and has been recently reported to occur in at least one-third of allo-HSCT patients and may be more severe in patients with complications. Indeed, depression influences quality of life and constitutes a risk factor for early post-transplant mortality. Anxiety and depression have also been associated with slower immune recovery following transplantation.

Growing evidence starts to highlight a role for the immune system in NC. Indeed, several central nervous system (***CNS***) autoimmunity models have demonstrated that cytokines orchestrate T cell-mediated immune responses. Proinflammatory cytokines such as IFNγ, TNFα, IL-1β, IL-6, and IL-12 are critically involved in the initiation and amplification of the local immune response in the CNS, which is counter-balanced by upregulation of anti-inflammatory cytokines such as IL-10.

Using an aGvHD murine model, *Hartrampf et al.* recently evidenced infiltration of donor T cells in the CNS of mice undergoing allo-HSCT. Importantly, they also observed significant neurocognitive deficits, which appeared in early stages of aGvHD. Despite the lack of mechanistic explanations, these findings present evidence that the CNS is a potential target of alloreactive T cells and suggest

that aGvHD could be a contributing factor in neurological and cognitive deficits, but also depression, following allo-HSCT. Using a rhesus macaque transplant model, *Kaliyaperumal et al.* confirmed that the brain is a target of GvHD, predominantly mediated by integrin-expressing $CD8^+$ T cells. However, little is known about the mechanisms used by these cells to infiltrate the brain.

One of these mechanisms could be the production of IL-27 by monocytes/macrophages, dendritic cells (both donor and resident cells) and microglial cells (brain resident immune cells). Indeed, IL-27 is a pleiotropic cytokine, belonging to the IL-6 superfamily and being at the same time initiator of Th1- and Th17-type immune reactions and a barrier against excessive inflammation, by promoting IL-10 production by Type 1 Regulatory cells (***TR1***, defined as $FoxP3^-IL\text{-}10^+$). IL-27 binds to a specific receptor, IL-27R, which is mainly expressed on immune cells, including T_{regs}. A role for IL-27 in autoimmune inflammation of the CNS has been well described in experimental autoimmune encephalomyelitis. In this model, lack of IL-27R has been demonstrated to enhance EAE severity by augmenting both Th1 and Th17 inflammatory reponses. However, little information is available on IL-27 and GvHD, especially CNS GvHD.

Current strategies to dampen GvHD are aimed at modulating the alloreactivity of donor cells outside of the CNS. In this future project, we propose a more global approach that aims at modulating the immune system, not only in the periphery, but also in the CNS in order to prevent neurological and cognitive deficits and ultimately improve quality of life of the patients. Specifically, the main goal of this project is to better understand how the IL-27 signaling pathway will regulate T-cell alloreactivity in the CNS and how this will be translated into behavior. This project will be separated in 3 objectives: 1) Determination of the role of IL-27 in CNS aGvHD pathogenesis, 2) Impact of an antibody-based treatment on CNS aGvHD, and 3) Impact of an antibody-based treatment on cGvHD.

1. Determination of the role of IL-27 in CNS aGvHD pathogenesis

To determine whether IL-27 plays a significant role in CNS GvHD, IL-27 production in the CNS during aGvHD will be first assessed by flow cytometry and PCR analyses for IL-27 secretion. However, determination of the origin of IL-27 in the CNS is crucial for the understanding of pathophysiology. To determine the relevant contribution of donor and recipient cell populations, IL-27 knock-out mice will be used. We plan to process brains from GvHD mice by flow cytometry, PCR analyses, and histology. This will allow determining the origin of IL-27 production and its influence on immune cell infiltration and cytokine production within the brain of recipient mice.

However, IL-27R is also highly expressed on immune cells, including the microglia. By using IL-27R$^{-/-}$ mice, we will be able to determine, by flow cytometry and PCR, which immune subsets are dependent of IL-27R signaling for CNS GvHD development. IL-27R$^{-/-}$ will be used as donor and recipient in different experiments to again study the dependency of both CNS resident cells (like microglia) and donor cells (mainly T cells).

Importantly, as exposed earlier, T_{regs} are mandatory to maintain peripheral tolerance. Alterations in T_{regs} are also believed to play an important role in autoimmune neuroinflammatory diseases, as highlighted by EAE models. Moreover, IL-27 limits the T_{reg} cell population by inhibiting FoxP3 upregulation *in vitro*. Indeed, transgenic mice overexpressing IL-27 had decreased T_{reg} frequencies and developed spontaneous inflammation. To elucidate the mechanism by which IL-27 could limit or promote CNS GvHD, we propose to study the effect of IL-27 on this regulatory population.

However, even if we observe a beneficial effect on T_{reg} cell expansion, we must carefully investigate the effect of IL-27 and/or IL-27R signaling depletion on IL-10 production by both donor and recipient cells. Indeed, as exposed earlier, IL-27 is a potent inducer of IL-10 production by TR1 cells. Furthermore, IL-10 is critical to dampen neuroinflammation since depletion in IL-27 could result in more severe neuroinflammation despite T_{reg} expansion.

2. Impact of an antibody-based treatment on CNS aGvHD

A more clinically relevant approach will also be investigated by testing an anti-IL-27p28 antibody. This antibody is specific for the cytokine IL-27 since it recognizes its specific p28 sub-unit. We, first, propose to treat recipient mice with two injections of this antibody at D0 and D+6 post-transplant to determine how inflammation in the CNS is affected by looking at number of cells (T cells, microglial cells, etc…) and cytokines produced (IFNγ, TNFα, IL-1β, IL-6 and IL-10). T_{reg} cell expansion and IL-10 production by TR1 cells will also be investigated.

3. Impact of IL-27 on cGvHD

Recently, IL-27 was found to alleviate lung fibrosis both *in vitro* and *in vivo*. Indeed, proliferation, differentiation and collagen synthesis by TGF-β1-treated lung fibroblasts was significantly decreased in the presence of IL-27 [413]. An experimental fibrosis model induced by bleomycin confirmed this observation. Indeed, increased IL-27 expression in bleomycin-induced pulmonary fibrosis was noted. However, IL-27 treatment could alleviate pulmonary fibrosis and

increase the survival of mice. Indeed, these observations were explained by IL-27 inhibition of the development of $CD4^+$ Th17, $CD4^+$ Th2 T, and $CD4^+$ T_{reg} cells. Production of IL-17, IL-4, IL-6, and TGF-ß were also decreased. Finally, IL-27 induced the production of IL-10 by $CD4^+$ T cells [414].

In this part, we propose to study the implication of IL-27 in cGvHD (both in the periphery and in the CNS) by performing antibody-based and transgenic murine experiments, by using different cGvHD models. CNS cGvHD will be investigated as explained earlier, while peripheral cGvHD will be investigated by harvesting different cGvHD targets such as the liver, skin and lungs. We will look at the different T-cell subpopulations, including $CD4^+$, $CD8^+$ and T_{regs}. Pro-inflammatory and anti-inflammatory cytokines will be measured by ELISA, PCR and flow cytometry. Fibrosis will be studied by immuno-histology. Survival will also be investigated by using both transgenic and antibody-based approaches.