"TLR7 activation by LDV / R848 (Resiquimod) prevents acute graft versus host disease and cooperates with anti-IL-27 antibody for optimal protection"

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Abstract
Graft-versus-host-disease remains an important complication of allogeneic hematopoietic cell transfer. We studied immune suppression by LDV/R848-TLR7 stimulation in acute GVHD mouse models. Our results have shown that TLR7 activation inhibits cDCs and allo responsiveness of T cells. Both inhibitions are dependent on type I IFN. This suppressive effect seems to hamper the GVHD initiation without affecting long-lasting implantation of donor T cells. In addition, GVHD prevention is associated with a decrease of inflammatory cytokine production and a reduction of organ lesions. We have also found that donor and host Treg cell numbers were increased in recipient mice and that their elimination compromised survival and exacerbated morbidity in R848-treated mice. When aGVHD was more severe, an anti-IL-27 blocking antibody was necessary to optimize the R848 protection by an upregulation Treg cell level. We conclude that R848-TLR7 stimulation modulates multiple aspects of GVHD and offers pote...

Document type: Thèse (Dissertation)

Référence bibliographique

Gaignage, Mélanie. TLR7 activation by LDV / R848 (Resiquimod) prevents acute graft versus host disease and cooperates with anti-IL-27 antibody for optimal protection. Prom. : Van Snick, Jacques ; Coutelier, Jean-Paul

Available at: http://hdl.handle.net/2078.1/197054

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TLR7 activation by LDV / R848 (Resiquimod) prevents acute graft versus host disease and cooperates with anti-IL-27 antibody for optimal protection

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Thèse présentée en vue de l’obtention du grade de Docteur en Sciences Biomédicales et Pharmaceutiques

-Avril 2018-
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This thesis was supported by PhD fellowship from FRIA-FNRS, UCL (bourse du patrimoine) fellowship and Joseph Maisin foundation.
Je tiens tout d'abord à remercier mon promoteur Jacques Van Snick. Vous m'avez donné envie de poursuivre mon sujet de mémoire et d'entamer cette thèse de doctorat. Merci pour votre soutien, encouragements ainsi que votre présence qui a été inestimable dans ce projet ! Je remercie également Jean-Paul, mon deuxième promoteur, de m'avoir accueilli dans son laboratoire et d'avoir veillé à ce que tout se déroule bien !

Je tiens particulièrement à remercier mes 2 promoteurs pour la liberté et la confiance qu'ils m'ont accordées et pour les critiques judicieuses.

Merci aux membres de mon comité d'accompagnement pour leurs conseils et encouragements.

Je remercie particulièrement Reece, Pamela et Perrine pour leur aide précieuse à ce projet et leur amitié plus que nécessaire dans ces longues journées de labo !

Merci aussi à mes collègues-amies (Emilie, Camille, Elisabeth, Elsa, Muriel, Lorraine, Astrid, Magali, Sarah, Nadia et Laure) pour leur présence au quotidien permettant ainsi des journées légères et agréables.

Je remercie mes parents et Marine. Ils ont également participé à ce travail mais « derrière les caméras ». Ils ont été des coaches de vie indispensables (soutien, encouragements, nourrie,...). Et très récemment, ils prennent soin de mon « bouchon », me faisant des petits comptes rendus illustrés !

Enfin je remercie mon conjoint, Bastien, et sa technique infaillible pour me déconnecter de boulôt ! Sa présence mais aussi son humour et sa bonne humeur, m'ont permis de prendre du recul sur ma thèse, ce qui, parfois, était plus que nécessaire.
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Summary

Allogeneic haematopoietic cell transplantation (HCT) is a potent immunotherapy with curative potential for several haematological and non-haematological disorders. Its success is however hampered by acute graft-versus-host disease (GVHD) that occurs when donor T cells mount a strong immune response against the recipient after activation by histocompatibility antigens. In spite of improvements in donor and recipient selection, in conditioning regimens, in GVHD prophylaxis and treatment and in prevention of infectious complications, GVHD remains a major impediment for the expansion of the therapeutic use of Allogenic-HCT (Allo-HCT).

Infectious agents as well as components of the innate immune response such as toll-like receptors (TLRs) are significantly implicated in GVHD development. We have discovered that mouse infection with nidovirus LDV improves survival in GVHD induced in non-conditioned B6D2F1 mice by transplantation of B6 parental spleen cells. This protection correlated with an inhibition of allo-antigen presentation by conventional dendritic cells (cDCs) and with a hypo-responsiveness of T cells in allo-reactivity. Both inhibitions were type I IFN-dependent.

As LDV activates TLR7, we tested TLR7 ligand R848, Resiquimod, in the same GVHD model and observed complete survival if donor and recipients were treated before transplantation. We also observed that T cell allogeneic responses and alloantigen presentation by cDCs collected from mice treated for 48h with R848 were inhibited in mixed lymphocyte cultures (MLC). These inhibitions were dependent on IFNAR-1 signaling. We found that, in transplanted mice, R848 decelerated, but did not abrogate, donor T cell implantation and activation. It decreased plasmatic Th1 cytokines, IFNγ and
IL-27, while upregulating active TGF-β1 production. In addition, Foxp3+ Treg cell numbers were increased in donor and recipient mice and their elimination partially impaired R848 protective activity.

A correlation between an increase in Treg and GVHD prevention has already been described in our laboratory by Reece Marillier [1] who found that blockade of IL-27p28 completely prevented aGVHD in the B6→B6D2F1 parent to F1 model and this correlated with upregulation of Tregs. The importance of IL-27 in aGVHD was also highlighted in a full allogeneic GVHD model (B6 donor and lethal irradiated BALB/c recipients). In this study performed by Dr. Ludovic Belle in the laboratory of Dr. William R. Drobyski (Department of Medicine, Medical College of Wisconsin, Milwaukee, Wi, USA), and to which I participated, we showed that targeting of IL-27 limited aGVHD by augmenting Treg reconstitution and stabilized Foxp3 expression. However, in this aggressive model, the anti-IL-27 antibody induced only 50 % of survival.

Finally, I observed that R848 also strongly improved survival of lethally irradiated BALB/c recipients of B6 grafts and this also correlated with an upregulation of CD4 and CD8 Foxp3+ Tregs that could be further increased by IL-27 inhibition. The anti-IL-27-R848 combination showed strong synergy in preventing disease in the B6 → B6D2F1 model when recipients were sublethally irradiated.

In conclusion, R848-TLR7-ligand modulates multiple aspects of GVHD and could offer potential for safe allogeneic bone marrow transplantation that can be further optimized by IL-27 inhibition.
List of abbreviations

aGVHD: acute graft versus host disease
AML: acute myeloid leukaemia
APC: antigen presenting cell
BM: bone marrow
cDC: conventional dendritic cell
CIBMTR: Center for International Blood and Marrow Transplant Research
CpG-ODN: cytosine-phosphorothioate-guanine oligodeoxynucleotide
DC: dendritic cell
GVHD: graft versus host disease
GVL: graft versus leukaemia
GVT: graft versus tumor
HLA: human leukocyte antigen
HSC: hematopoietic stem cell
HCT: hematopoietic cell transplantation
IDO: indoleamine 2,3-dioxygenase
IL-1: interleukin 1
LDV: Lactate Dehydrogenase-Elevating Virus
LPS: lipopolysaccharide
MLC: mixed lymphocyte culture
MSC: mesenchymal stromal cells
NK cells: natural killer cells
NLR: nod-like receptors
PAMP: pathogenic associated molecular pattern
PB: peripheral blood
PBCT: peripheral blood cell transplantation
PRR: pattern-recognition receptor
pDC: plasmacytoid dendritic cells
RORγt: RAR-related orphan receptor gamma
SAA: serum amyloid A
STAT4: signal transducer and activator of transcription 4
TBI: total body irradiation
TLR: toll-like receptor
TGFβ: transforming growth factor beta
TNFα: tumor necrosis factor alpha
UCB: umbilical cord blood
UCBT: umbilical cord blood transplantation
INTRODUCTION
Introduction

1. Hematopoietic cell transplantation

The first human bone marrow transfusion was performed in 1939 in a patient with aplastic anemia, a disease characterized by bone marrow failure. The patient was treated with daily intravenous bone marrow injection in an attempt to restore leukocyte and platelet levels [2]. This technique was studied and optimized in animal models in order to cure individuals with bone marrow failure induced by atomic bombardment during World War II [3]. In 1957, E.D Thomas and colleagues described a revolutionary treatment for patients with malignant diseases. This treatment was to inject bone marrow cells from healthy donors to patients having undergone total body irradiation (TBI) [4]. However, only two patients survived and 4 others died 100 days post transplantation. They were confronted to a histocompatibility antigen issue of which very little was known at that time. It was only in the middle of the 1960s that the first tests to compare human leukocyte antigen (HLA) were developed in order to verify whether donor graft HLA was similar to the recipient HLA [5]. In the seventies, HCT was used as a therapy against non-malignant and malignant hematologic disorders [6] and later, HCT was also performed on patients with solid tumor [7, 8] or with autoimmune diseases such as severe multiple sclerosis [9, 10]. Nowadays, HCT is a potentially curative therapeutic approach used for a large number of disorders: in 2014, 21 042 HCT were performed in the US [CIBMTR] and 40 829 HCT in Europe [11].

HCT results will depend on several factors such as the treated disease, the age of the patient as well as the HCT source and its conditioning. The conditioning
regimen prior HCT is an important step, which has the purpose of eradicating tumor burden and to suppress the patient immune system in order to avoid graft rejection. Different conditioning level intensities were developed and according to the disease and characteristics of the patient, the regimen has to be adapted.

The initial conditioning performed was total body irradiation (TBI) given in a single dose leading to ablation of the haematopoietic system [12, 13]. The advantage of high dose of TBI is the reduction of relapse risks. Despite of its required use against diseases such as acute myeloid leukemia (AML), such a treatment leads to severe side effects in target organs (intestine, liver, lung) and is not appropriate for children and patients older than 50 years [14]. To increase the anti-tumor activity and facilitate engraftment, different chemotherapies have been elaborated and added to the TBI regimen. The best known is Cyclophosphamide, which has antineoplastic and immunomodulatory properties [6]. Other agents such as Etoposide [15], Melphalan [16] and Busulfan [17] were also combined with TBI. To avoid toxicities associated with high-dose of TBI and widen the myeloablative treatment to patients that cannot undergo TBI, high-dose of chemotherapy has been developed without radiotherapy. The Cyclophosphamide-Busulfan combination was reported to decrease transplant-related mortality and an increase in disease-free survival compared to TBI regimen in AML patient [18].

At the end of the seventies, Weiden and colleagues observed that leukemic patients who developed graft versus host disease (GVHD) after allogeneic HCT had improved relapse-free survival, indicating the effect of allogeneic HCT against malignant cells [19]. This phenomenon called graft versus leukemia (GVL) led physicians to develop less aggressive conditioning regimen such as reduced-intensity/toxicity conditioning (RIC) and nonmyeloablative regimens.
These techniques used the same agents and radiotherapy than myeloablative regimen with lower concentration and level, respectively. In addition to these agents, Fludarabine, a purine analog, and/or anti-thymocyte globulin (ATG), a polyclonal antibody, are fluently administrated during a RIC procedure and maintain GVL effect without increasing GVHD [20-23]. Thanks to the toxicity reduction, those techniques became accessible to older individuals or comorbidity patients [14].

Depending on the severity and the disease type, autologous HCT (auto-HCT) or allogeneic HCT (allo-HCT) will be preferred (Figure 1).
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Autologous HCT involves the use of the patient's own hematopoietic cells to re-establish the hematopoietic system after conditioning. Allogeneic HCT requires hematopoietic cell transplantation from a donor to another person. The term “related HCT” means graft from the same family background whereas unrelated involves donor graft identified in an HCT registry. Finding a donor with nearly identical histocompatibility antigen genetics (matched donor), is a difficult process. The only optimal feasible case is the transplant between homozygote twins and the appropriate term is syngenic transplantation. Nevertheless, donors who have 8 HLA (HLA-A, -B, -C, and DRB1) identical alleles to the patient are considered as matched donors. The probability to have an HLA-matched sibling donor is less than 30%. In certain African-American ethnic groups the likelihood can decrease up to 20% [24]. Therefore, for patients without matching donors, alternatives are mismatched unrelated donor, haploidentical related donor, and umbilical cord blood (UCB) transplantation (discussed below). A commonly used strategy is to obtain a mismatched unrelated donor if he matches at least for 7/8 HLA alleles. More than one mismatch in unrelated graft increases significantly the risk of transplant-related mortality [25]. Another option is haploidentical transplants. In haploidentical transplantation, the donor comes from the same family background (parent, child) and shares at least 50% of match. Those 2 last strategies increased the allo-HCT feasibility. However, because of the mismatch, the risk to generate GVHD is more important [26]. The allo-HCT is necessary for aggressive diseases such as acute leukemia which require GVL reaction. This therapeutic approach significantly increases the non-relapse mortality [CIBMTR].

There are different sources of HSC. In 1957, the first HCT has been performed with bone marrow [4]. Thirty years later, with the availability of recombinant granulocyte-colony stimulating factor (G-CSF), peripheral blood (PB) became
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also a source of hematopoietic precursors [27]. Two decades ago, umbilical cord blood transplantation (UCBT) also emerged as alternative source of hematopoietic stem cells [28].

Unlike bone marrow, PB graft results in a higher proportion of progenitor stem cells as well as T cells leading to a better yield [29, 30], which results in a faster engraftment and in a lower risk of graft failure [31]. However, some studies reported a higher risk to develop chronic GVHD owing to the proportion of T cells, which is significantly higher in PB [31-33]. Although the administration of G-CSF could raise a risk to the donor health, the PB transfer techniques do not require donor anesthesia as for HSC from BM. The last 2 decades, PBC transplantation has been more widely used than BM transplantation [CIBMTR][34]. However, when the patients undergo mismatched unrelated donor graft, BM graft is preferred to avoid chronic GVHD [35].

Umbilical cord blood transplantation (UCBT) has emerged as a promising strategy for several reasons among which the higher clonogenic potential of HSC [36]. As the immune cells come from extraembryonic tissues, they have naïve immune properties such as high permissibility for HLA leading to important mismatched, 4–6/6 HLA alleles (HLA-A, HLA-B and HLA DRB1) [37, 38]. UCBT offers a substantial alternative for patients without suitable matched graft because of their diverse ethnic background for instance [39, 40]. In addition, UBCT involves lower risk of GVHD [41]. For those reasons, the technique is also more adapted for children needing HCT [42]. Another advantage is that UCB is easy to collect compared to BM and, today, UCB units are collected worldwide (NETCORD members span all continents) increasing the biobanks content worldwide (BMDW). However, UCBT are associated with a higher rate of engraftment failure and an increased risk of infection due the
naive nature of the cells since they have never been exposed to pathogens [43]. Another disadvantage is the low number of stem cells present in UCB, which limits the success of transplantation in to adults [44]. Nevertheless, different approaches have been applied to increase the low amount of stem cells such as ex vivo expansion of the cord unit and the use of double cord blood units [45]. The latter preserves the GVT and accelerates graft implantation [46, 47]. However, the use of single versus double UCBT has not been tested in a phase III study in adults and remains controversial [42].

Despite of improvement of allo-HCT strategies such as reduced-intensity conditioning and optimal graft source selection, the allo-HCT is regularly hampered by graft versus host disease (GVHD), which leads to high morbidity and mortality.

2. Graft versus Host Disease

Graft versus host disease was initially reported by Barnes and colleagues in 1956. They worked on mice with acute leukemia and two groups were conditioned before bone marrow transplantation. One group was treated with a syngeneic bone marrow and most of the mice died from leukemia relapse. The other group was transplanted with allogeneic bone marrow cells and mice did not develop disease relapse; however they all died from a “wasting syndrome”. For the first time, these experiments highlighted a straight link between the capacity of engrafted immune cells to prevent leukemia relapse, GVL, and the ability to strongly react against the recipient and induce GVHD [48]. A decade later, Billingham defined GVHD as a disease induced by a graft that must contain immunologically competent cells; a recipient expressing tissue antigens that are not present in the transplant donor; and a recipient who is not able to mount an effective response to reject the transplanted cells
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According to the Center for International Blood and Marrow Transplant Research (CIBMTR), 8-9.5% of mortality following allo-HCT is due to GVHD in 2014.

Two forms of GVHD exist, an acute and a chronic, and they involve different pathological mechanisms. Acute GVHD (aGVHD) induces a strong inflammatory response whereas chronic GVHD (cGVHD) presents autoimmune and fibrotic features. In a simplified context, GVHD is defined as acute if it develops in the 100 days following the HCT and becomes chronic after that time [50-52].

a. Pathophysiology

The pathophysiology of aGVHD has been described by Ferrara and colleagues as a three-phase process (Figure 2): an induction phase, a differentiation/activation phase and an effector phase [53]. The initial phase is generated by an important inflammatory environment that activates antigen presenting cells (APCs). This inflammatory response is caused by the treated disease of the patient and the conditioning regimens prior to allo-HCT and leads to target tissue damages. The target organs are mainly intestinal tract, liver, lung and skin. The tissue destruction leads to a "cytokine storm", which results from production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin 1 (IL-1) and IL-6 [54]. Those cytokines activate APCs from the patient and from the donor. In addition, the inflammatory environment is also maintained by the release of pathogen-associated molecular pattern molecules (PAMPs) from gut microbiome since intestinal epithelium is dramatically affected by conditioning. Once activated, APCs present alloantigens to the donor T cells which recognize them as foreign [55, 56]. This interaction induces the second phase, activation and
differentiation of donor T cells. Activation of CD4 T cells results from their TCR-MHC-II binding and their stimulation by pro-inflammatory cytokines such as TNFα and IL-1, whereas the CD8 donor lymphocytes are activated by MHC-I binding and characterized by the presence of perforin, granzyme as well as Fas-Ligand. Once activated, T lymphocytes differentiate and expand. The effector phase is characterized by cytolytic activity of T cells inducing target organ damage. In addition, due to this inflammatory environment, other immune cells such as neutrophils, natural killer cells (NK) and macrophages are enrolled and amplify this process leading to an endless reaction [53].
**Introduction**

**b. Innate immune receptors in aGVHD**

The microflora, endo- and epithelial cells as well as components of the innate immune response are significantly implicated in GVHD development. Conventionally, the conditioning regimen induces tissue damage including a
loss of epithelial barrier function. This permeability allows translocation of microflora elements called pathogen-associated molecular patterns (PAMPs) which stimulate pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs) or nod-like receptors (NLRs) mostly expressed on APCs. Following PRR-ligation, APCs are activated and produce pro-inflammatory cytokines and present antigens to donor T cells. The APC activation can also be amplified by damage-associated molecular patterns (DAMPs) which are endogenous non-infectious molecules such as ATP released by damaged cells (Figure 3) [57-59].

Figure 3. Schematic overview of the initiation phase of aGVHD. During the toxic conditioning regimen with total-body irradiation and/or chemotherapy, the destruction of intestinal epithelial cells leads to the loss of the epithelial barrier function. The subsequent translocation of luminal bacteria as well as the release of endogenous danger molecules such as adenosine triphosphate (ATP) and uric acid result in the production of pro-inflammatory cytokines. Activated host and/or donor antigen-presenting cells then prime allo-reactive donor T cells, which perpetuate aGVHD. TLR-toll-like receptor; APC-antigen-presenting cell; DAMP-danger-associated molecular pattern; TNF-tumor necrosis factor; IL-interleukin; NOD2-nucleotide-binding oligomerization domain; NLRP3, NACHT, LRR, and PYD domains-containing protein 3. [55]
The involvement of intestinal microflora in GVHD was already reported in 1974. Lipopolysaccharide (LPS) present in Gram-negative bacteria, was associated with a severe GVHD in irradiated mouse models [60]. These results have been supported by the utilization of germ-free mice leading to less severe GVHD and antibiotic treatment that mitigated intestinal GVHD [61, 62]. In parallel, antibiotic administration prior to allo-HCT in patients reduces bacterial flora and confers protection against aGVHD. Therefore, prophylactic antibiotic treatment is commonly used in prevention in many transplantation centers worldwide [63-65].

In addition to bacteria, fungal populations (mycobiome) could interfere with the GVHD development. In humans, colonization of intestine by Candida spp leads to a severe GVHD as compared to uncolonized patients [66].

On the other hand, certain commensal bacteria such as Lactobacilli seem to play a beneficial role in mouse GVHD pathogenesis. Elimination of this species from the mouse flora before allo-HCT aggravates GVHD whereas its reintroduction has the opposite effect [67]. Therefore, the administration of large-spectrum antibiotics as prophylaxis could have unwanted effects. A survey recently reported that patients treated with antibiotics prior to aHCT developed more severe aGVHD [68].

As microflora modulates the aGVHD course, scientists have attempted to understand the implication of TLRs in this pathology. TLRs are transmembrane proteins expressed in surface membrane for TLR1, 2, 4, 5 and in lysosome membrane for TLR3, 7/8 and 9. The most-studied TLR is TLR4 that can be strongly stimulated by LPS. Following LPS translocation, TLR4 activation contributes to severe GVHD via tumor necrosis factor-α (TNFα) secretion. The critical involvement of LPS-TLR4 was confirmed with an LPS antagonist that decreases GVHD severity [58, 69]. Nonetheless, TLR4
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stimulation does not seem to be required for GVHD initiation and under certain conditions, TLR4 activation seems to have a beneficial role against the disease [70, 71]. TLR4 involvement remains unclear in human. One study reported that reduction of TLR4 function due to the SNP mutation was associated with less severe GVHD. However, these results could not be replicated [72, 73].

TLR9 is also known for its involvement in aGVHD pathogenesis. This TLR detects cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG-ODN) motifs. Mice deficient for TLR9 develop a mitigated GVHD [70]. In addition, mice repeatedly treated with CpG-DNA after allo-HCT develop a rapid lethal GVHD [74]. Until now, no evidence in human illustrated the TLR9 promoting effect on GVHD [75, 76].

On the other hand, TLR5 has been associated with immune regulatory response in GHVD development in the mouse, but opposite results were reported in human [77, 78].

TLR7/8 binds single-stranded RNA and induces anti-viral response. TLR7/8 contribution in GVHD pathogenesis differs according to the dose administered and the timing. Chakraverty and colleagues demonstrated that systemic exposure of R848, a TLR7 agonist, induces a massive recruitment of donor T cells in peripheral tissues leading to skin GVHD in the mouse [79]. Interestingly, the administration of 3M-001 (TLR7/8 agonist) prior to HCT protects mice against aGVHD in an indoleamine 2,3-dioxygenase (IDO) dependent pathway, whereas 3M-001 treatment after HCT aggravates GVHD [74, 80]. However, no study describes the potential implication of TLR7/8 in human GVHD.
Another family of PRRs related to GVHD pathogenesis comprises the cytoplasmic NLRs. NOD1 and NOD2 recognize peptidoglycans from bacterial cell wall. Investigations in mouse models highlighted the immunosuppressive effect of NOD2 in GVHD through its inhibitory effect on host APC function. This was supported by the exacerbation of GVHD in NOD2-deficient mice [81]. However, its implication in human GVHD pathology remains unclear [57].

c. Antigen presenting cells in aGVHD

It is now well established that APCs are important for the induction of GVHD. APCs have been intensively studied in order to determine the precise involvement of their subsets in order to use them for GVHD inhibition. These investigations have highlighted the critical implication of dendritic cells in this process [82].

DCs are the main APCs and are derived from hematopoietic precursors [83]. They are divided in 2 main populations, conventional and plasmacytoid. In the steady state, quiescent DCs capture self-antigens to present them at low rate to T cells in lymphoid organs. This leads to deletion of the corresponding T cells and maintains self-tolerance. Once activated by PRRs, pro-inflammatory cytokines or co-stimulatory factors, DCs become mature and their capacity of acquiring, processing and presenting antigen peptides is increased [84]. Unlike other APCs, DCs have the ability to return in T cell zones of lymphoid organs for optimal interactions with T lymphocytes [85].

Following HCT, both donor and recipient DCs are involved in GVHD, although their precise function in this process remains incompletely understood [82]. Host DCs are sufficient to prime donor CD4 and CD8 lymphocytes and initiate aGVHD when they are transferred into MHC-deficient recipients [86]. Zhang and colleagues have shown that following TBI, recipient DCs are still capable
to prime allogeneic T cells before disappearing [87]. In lymphoid tissue, mouse host DC population is totally replaced by donor DCs within two weeks, indicating that the transient persistence of recipient DCs is sufficient to initiate GVHD [82, 88]. This turnover was also suggested in human since DCs in skin are predominantly of donor origin 40 days after BMT [89]. An important aspect in the GVHD development is the maturation level of DC. Prevention of CD40 interaction of mature DC inhibits allogeneic T cell activation and affects GVHD course [90].

Regarding DC subsets, several investigations have been performed in order to elucidate their implication in GVHD. However, depending of the context and the mouse model, their functions can be different. Splenic plasmacytoid DCs expressing CCR9 have tolerogenic effects and are able to abrogate aGVHD [91]. In addition, GVHD development affects pDC maturation, leading to a suppressive immature pDC population [92]. On the other hand, Koyama and colleagues have shown that, in absence of conventional DCs, pDCs were sufficient to prime alloreactive T cells and mediated aGVHD [93]. Another specific DC subset, Langerhans cells, are also implicated in GVHD. In a mouse skin GVHD model, they were shown to be sufficient to generate aGVHD [94]. However, these cells are not required to initiate the disease [95].

Profound host APC depletion does not prevent aGVHD suggesting the participation of non-hematopoietic APCs in this process [96]. After TBI, epithelial cells in the gastrointestinal tract express MHC class II and costimulation molecules such as CD40, CD80, and CD86 due to the inflammatory environment. In this context, those cells present antigens as an integral part of their function and prime T cell generating aGVHD [97]. This concept is correlated with clinical studies where biopsy samples from patients demonstrate high expression of HLA class II on colonic non-hematopoietic
cells after transplantation [98]. As to donor DCs, they are able to initiate aGVHD in an MHC-class II-dependent pathway. However, donor DCs failed to induce CD8-dependent GVHD in a mouse model [99].

d. T cells in aGVHD

T cells are the main inducers of GVHD. In allo-HCT mouse models, both CD4 or CD8 alone are able to induce aGVHD. In human, the aGVHD is reduced in patients transplanted with CD8 T cell-depleted or CD4 T cell-depleted grafts [100]. Once primed by alloantigen, donor T cells will differentiate and follow a specific response pathway promoted by released cytokines. This process of generating differentiated effector T cells is determinant for defining the type of GVHD development (Figure 4).
T Helper (Th)1 response is largely involved in pathophysiology of aGVHD and particularly, plays a detrimental role in gastro-intestinal tract alterations [101, 102]. Differentiation of T cells into Th1 subset is induced by the production of IFNγ. IFNγ also prompts DCs to secrete IL-18 and IL-12 and the latter triggers the signal transducer and activator of transcription 4 (STAT4) pathway [103]. Th1 cells are identified by the activation of transcription factor T-bet which also inhibits Th2 differentiation [104]. IFNγ is important in the efferent phase
of aGVHD where its production is strongly increased. This process has been observed in both animal models and clinical studies [105-108]. IFNγ leads to APCs activation and to recruitment of effector cells such as monocytes and NK cells in target organs [109]. Some mouse studies have highlighted the importance of IL-18, which contributes to \( T_{H1} \) differentiation and T cell expansion [110, 111]. Furthermore, Tbet-deficient donor cells fail to induce a lethal aGVHD [112].

However, early \( T_{H1} \) differentiation seems to impair mouse aGVHD. Reddy and colleagues have shown that IL-18 treatment prior to allo-HCT alleviates lethal GVHD in a Fas dependent process [113]. Also, mice treated by IFNγ or IL-12 just before or during allo-HCT develop a less severe aGVHD [114, 115].

\( T_{H2} \) cells are generated by GATA-3 transcription factor and are mainly characterized by cytokines such as IL-4, IL-5, IL-10, IL-13 and by STAT6 pathway activation [103]. The type of GVHD response following a \( T_{H2} \) polarization depends on the mouse GVHD model. It was firstly described that donor \( T_{H2} \) cells mediated a chronic GVHD in a parent to F1 model [116, 117]. However, another study has shown in a full allogeneic model that donor cells deficient for IL-4 lead to a mitigated aGVHD [118]. This was supported by a delay of aGVHD when donor cells originated from STAT6 knock-out (KO) mice [119]. Therefore, these findings suggested that \( T_{H2} \) responses may also contribute to aGVHD.

In contrast to \( T_{H1} \)-mediated aGVHD, \( T_{H2} \) effector T cells affect preferentially liver and skin [119, 120]. This notion that \( T_{H} \) response in aGVHD differs according to the target organs is now well established [121].

Another T cell subset, \( T_{H17} \) effector T cells, was also found to be involved in aGVHD and mainly in skin and lung alterations in mouse models [119, 122].
TH17 differentiation, characterized by expression of the transcription factor orphan nuclear receptor (RORγt), is induced by IL-6 and TGF-β. IL-23 produced by macrophages contributes to the survival of these donor TH17 cells that secrete IL-17A, IL-17F, IL-21 as well as IL-22 [103]. In contrast to TH1, TH17 implication in aGVHD is not straightforward. Adoptive transfer with donor TH17 T cells has been shown to induce an aGVHD [122, 123]. However, donor T cell deficient for RORγt are still capable to induce a lethal GVHD, albeit less severe [112, 123]. On the other hand, Yi and colleagues have demonstrated that TH17 regulated TH1 donor cells since in absence of TH17 cells mice develop a severe TH1-mediated GVHD [124].

Several studies have also investigated the role of cytokines (IL-23, IL-21, IL-22) implicated in TH17 response in aGVHD mouse. Overall, mice transplanted with donor cells deficient for any of these cytokines showed a reduced aGVHD [125].

The identification of T regulatory cells (Tregs), characterized by the expression of the transcription factor Foxp3 and induced by IL-2 and TGF-β, has led to intensive research with the purpose to induce tolerance. In an aGVHD context, Tregs are mainly repressed due to induction of other TH responses. Different studies have shown that blocking cytokines involved in GVHD pathogenesis allowed for an increase of Treg reconstitution in mouse models. The inhibition of IL-6 signaling attenuates the severity of aGVHD with an increase of Tregs [126]. More recently, the blockade of IL-27p28 was shown to completely prevent aGVHD, and this correlated with an upregulation of Tregs [1]. On the other hand, increasing the Treg ratio in donor graft abrogated aGVHD in the mouse [127]. Due to this potential induction of tolerance, therapeutic approaches such as adoptive transfer are being developed. In a XenoGVHD model (human PBMCs transfer to NSG mice), the
infusion of enriched human Tregs delayed the occurrence of xenogeneic GVHD without inducing toxicity in NSG immune-deficient mice [128].

Regarding the implication of CD8 cytotoxic T cells in GVHD, very little is known. Like CD4 T cells, CD8 T cells can also differentiate along similar type 1 and type 2 pathways based on IFN$\gamma$ and IL-4, -5, -10 and -13 production, respectively [121]. Interestingly, a study has shown that administration of CD8 T cells differentiated to Tc1 or to Tc2 in vitro with IL-12 or IL-4 respectively, induce less severe GVHD while preserving GVL activity [129]. Recently, CD8 + cytotoxic Tc17 cells were identified and seem to have a pro-inflammatory effect and contribute to GVHD generation [130].

e. Risk factors

GVHD development is promoted by different factors such as conditioning regimen, graft source, ineffective prophylaxis, HLA disparity, non-HLA gene polymorphism, male versus female donor and older age of donor and recipient.

GVHD occurs when donor T cells mount a strong immune response against the host after minor and major histocompatibility antigen recognition. MHC class I molecules including HLA-A, HLA-B, and HLA-C are expressed at variable levels by all nucleated cells and present intra-cellular peptides to CD8 T cells. On the other hand, MHC class II molecules (HLA-DR, HLA-DQ, and HLA-DP) are mainly expressed by APCs (B cells, dendritic cells, macrophages) and present extra-cellular peptides to CD4 T lymphocytes [131].

HLA alleles are highly polymorphic and the incidence of acute GVHD is directly related to the degree of mismatch between donor and recipient HLA proteins. Therefore, the ideal situation is to transplant HSC of a donor whose HLA-A,
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HLA-B, HLA-C and HLA-DRB1 8/8 allele pairs match those of the recipient [132, 133].

Recently, a study demonstrated the implication of MHC class I chain-related gene A (MICA) in GVHD development. MICA is a non-conventional MHC I molecule, which is also highly polymorphic and is a stress-induced glycoprotein expressed mainly on epithelia [134].

Gene polymorphism of minor histocompatibility antigens (HA) can also lead to an aGVHD since 40% of patients transplanted with a 8/8 HLA-matched donor graft still develop the disease. The most studied HAs are HA-1, HA-2, HA-3 and HY. HA-1 and HA-2, are expressed mainly on hematopoietic cells, which increases also the GVL effect, whereas HY and HA-3 are expressed on all tissues [135]. HY refers to a minor antigen from Y chromosome and becomes a risk factor if male patients are transplanted with a female graft. Therefore, since 2 decades the frequency of female donor graft transplanted to male recipient has significantly decreased [136, 137]. Another aspect to consider is multiple pregnancies for female donors. Women develop T and B allogeneic memory cells against paternal antigens due to the cell placental exchange between mother and fetus. Therefore, the more pregnancies the donor has had the more memory allogeneic lymphocytes will be present, which increases the risk of GVHD development [138, 139].

At the end of the nineties, Middleton and colleagues highlighted the potential implication of non-HLA gene polymorphism on GVHD initiation [140]. The best-known gene polymorphisms are single-nucleotide polymorphisms (SNPs). SNPs are individual variations of a DNA sequence which result in differences in amino acid coding between recipients and donors creating non-HLA minor histocompatibility antigens. It was estimated that 1% of genome-wide difference between recipient and HLA-matching donor is associated with
Introduction

a 20% increase in the risk of severe acute GVHD [141, 142]. On the other hand, SNPs present in cytokines, chemokines, costimulatory molecules involved in GVHD pathophysiology are also associated with a greater risk of acute GVHD.

For instance, polymorphisms in cytokine genes such as TNF-α, IL-1, IFNγ, which leads to an increase of cytokine level, can be predictive for disease development [141].

f. Diagnosis

As previously stated, acute GVHD was defined to occur prior to day 100, whereas chronic GVHD if it occurs after that time [50-52]. However, the reality is not as straightforward. aGVHD appearance can be delayed due to reduced-intensity conditioning or prophylaxis treatment [143, 144].

Clinical manifestations of acute GVHD occur within target organs such as skin, liver and gastrointestinal tissue [144]. Skin is more frequently affected and is usually the earliest organ involved. The injuries are characterized by a pruritic maculopapular rash that can cover the whole body and, in severe cases, the skin can ulcerate [144-146]. The consequences of aGVHD in the gastrointestinal tract are diarrhea as well as vomiting, anorexia and abdominal pain [145-147]. In severe cases, the patients develop ulcers within intestinal mucosa [148].

Regarding the liver, lesions caused by GVHD are similar to damages generated by chemo/radio-therapy regimen prior to HCT and infections, which can complicate the diagnosis. The common clinical features of hepatic GVHD are endothelialitis, lymphocytic infiltration of the portal areas and bile duct destruction [149, 150].
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The diagnosis of aGVHD is assessed according to the target organ damaged and the severity of the disease is graded clinically following the intensity of the lesions. Grade I acute GVHD is considered to be mild and involves moderate skin problems (less than 50% of total body rash) whereas grade II (moderate), III (severe) and IV (very severe) include skin, gut and liver damages. aGVHD reaching grade III becomes really complicated to treat and leads to a poor long-term survival [151]. However, prognosticating aGVHD outcome is a complex task. Skin and liver biopsies can be performed to strengthen the diagnosis. However, these techniques remain controversial and liver is rarely biopsied because of thrombocytopenia early after transplant increasing the risks of this procedure [149, 150, 152]. Since a few years, biomarker panel analysis was elaborated to confirm the GVHD prognostic. The patients with plasmatic IL-2-receptor-a, TNF receptor-1, IL-8 and hepatocyte growth factor, are prone to develop aGVHD [153]. More recently, suppression of tumorigenicity 2 (ST2), an IL-33R involved in TH2 response, has also been identified as an important biomarker predicting aGVHD development [154, 155]. The use of biomarkers for aGVHD diagnosis remains under investigation.

g. Treatments

Prophylaxis - GVHD prevention

The prophylaxis strategy used in order to decrease the GVHD risk was initially described in 1986 by Storb and colleagues. This preventive approach is usually implemented with patients undergoing myeloablative regimen and is mainly based on T cell inhibition [156, 157]. The commonly used prophylaxis regimen includes a combination of a calcineurin inhibitor and a short course of methotrexate (MTX) [158]. Calcineurin inhibitors such as cyclosporine and tacrolimus have as function to inhibit the T cell signaling pathway while MTX
blocks DNA and RNA synthesis leading to diminished T cell proliferation [159]. Clinical trials have reported the superiority of the combinations in GVHD prevention compared to each agent alone [160, 161]. Among the different combinations, tacrolimus-MTX significantly reduced aGVHD significantly more than cyclosporine-MT; however, the long-term survival rate remained unchanged [162, 163].

Another combination used as GVHD prophylaxis is Calcineurin inhibitors and mycophenolate mofetil (MMF), which also inhibits T cell proliferation. Compared to MTX, MMF with calcineurin inhibitors, seems to slightly improve the quality of life. However, this needs to be confirmed [164].

More recently, sirolimus, also known as rapamycin, was used as another alternative agent in GVHD prevention. Rapamicin is an inhibitor of mTOR leading to impaired T cell signalling and DC functions [165-167]. Combined with tacrolimus, sirolimus showed promising results such as rapid engraftment, reduced aGVHD incidence and a decrease of transplantation-related toxicity [168]. A recent phase III clinical trial has confirmed the superiority of Sirolimus-tacrolimus versus tacrolimus-MTX [169]. However, Sirolimus was also associated with the appearance of sinusoidal obstruction syndrome/veno-occlusive disease, which involves destruction of sinusoidal endothelial cells and hepatocytes leading to ascites, tender hepatomegaly and jaundice [170, 171].

Other strategies based on T cell depletion were tested such as ex vivo T cell elimination or injection of T cell depleting antibody. Despite the strong prevention of GVHD development, those techniques significantly increase the risk of tumor relapse and infections [172-174].
Introduction

**GHVD treatment**

As previously described, aGVHD affects the skin, the liver and gastrointestinal tract. GVHD intensity is assessed by a grading system (grades I-IV) that depends on the lesion severity and the number of damaged organs [175]. The first line therapy against aGVHD is a corticosteroid treatment. Corticosteroids inhibit the inflammatory cytokine cascades and patients developing grade II to IV GVHD are usually treated with methylprednisone [176]. For patients developing grade I skin GVHD, the treatment can be performed locally with steroid creams such as triamcinolone or hydrocortisone. It is also the case for gastro-intestinal GVHD for which non-absorbable steroids are delivered by oral route [177].

Another approach to treat more specifically patients with gastro-intestinal and/or liver GVHD is steroid administration by intra-arterial route [178, 179], which improves the steroid recovery rate as compared to intravenous administration [180].

This first-line strategy leads to a durable response rate of only 40–60% [181-183] with a probability that is inversely proportional to the aGVHD severity. For patients who develop steroid-refractory aGVHD, a second-line therapy is considered; however the response rate with this treatment remains low [184].

The most frequently used second-line treatments are antibodies directed against GVHD-effectors such as ATG. Several monoclonal antibodies were also developed that recognize antigens on effector T cell surface. This is the case for Alemtuzumab, which binds specifically CD52 present on active human leukocytes or Visilizumab, a humanized monoclonal anti-CD3 antibody, which induces apoptosis of activated T cells. All those antibodies have shown some efficacy as second-line treatment; however, they considerably increase opportunistic infections [185-187]. Several therapies have also emerged using
Introduction

anti-IL-2-receptor (CD25) or anti-TNFα (etanercept) blocking antibodies. However, here also, infections remain the most significant side-effect [176].

Other strategies currently under investigation include Alefacept, a fusion protein including human LFA-3 extracellular domain and an IgG1 Fc portion, which blocks CD2–LFA-3 costimulation on memory effector T cells. Alefacept is already used in psoriasis [188] and first clinical trials have demonstrated a high response rate in steroid-refractory GVHD patients [189-191]. Sirolimus and MMF can also be used as second-line treatment. They have already shown some efficacy and are still under investigation (phase II clinical trials) [176, 192].

An alternative for treating steroid-refractory GVHD patients is the infusions of mesenchymal stromal cells (MSC). In vitro, MSC have the ability to suppress T lymphocyte response as well as activated APCs, natural-killer cells, and B cells [193]. Several studies have been performed with this approach, and globally, the protective effects shown in the first publications were only reproducible under certain conditions of age of the patient and of GVHD grade [176, 194].

Until now, no steroid-free treatment has shown clear superiority [195] and the strategy used depends on patient characteristics and physician preferences, which complicates data meta-analysis. Patients who were refractory to corticosteroid treatment, have a poor long-term prognosis with a mortality rate of approximately 70–80% [181, 196]. These statistics illustrate the urgent need of alternative therapies. Nevertheless, several investigations are ongoing and are currently in Phase I/II clinical trials. They become more specific for particular GVHD mechanisms as, for instance, cytokine modulation or adoptive regulatory cell therapy.
Studies on adoptive regulatory T cell therapy have shown interesting results. Infusion of native donor Tregs correlated with a lower incidence of aGVHD and an improvement of immune reconstitution [192, 197-199]. However, the procedures used to amplify Tregs and transfer them into patients are complicated and still need to be ameliorated [200]. These issues could be bypassed by IL-2 administration. Daily low-dose administration of IL-2 to SR chronic GVHD leads to an increase and durable Treg response improving cGVHD prognosis of more than 60% of patients [201]. In addition, ultra-low dose injection as GVHD prophylaxis after HCT induces Tregs expansion without preventing GVL activity [202]. However, in an experimental mouse model, low dose IL-2 administration to donor mice was not sufficient to protect against aGVHD [203]. Recently, a different approach that generated tolerogenic DCs has been developed by incubating human dendritic cells in vitro with IL-10. These DC-10 had the ability to induce type 1 regulatory T cells (Tr1) by a process depending on IL-10 and heme oxygenase-1 (HO-1). Gregori and colleagues elaborated an in vitro method to induce allo-specific Tr1 cells, using DC-10 [204, 205]. They have shown that in vitro, this allo-specific Tr1 had the ability to suppress allo-antigen specific effector T cells. A pilot clinical trial for adoptive transfer of donor Tr1 cells from DC-10 into patients undergoing HCT is under investigation. Preliminary results shown that adoptive Tr1 transfer after HCT improves immune-reconstitution without severe graft-versus-host disease manifestation [206].

The cytokine most studied in human GVHD therapy is IL-6 and specific treatments against it begin to emerge. Tocilizumab is a humanized anti-IL-6 receptor antibody blocking IL-6 signaling and, recently, showed promising results in phase I trials when used as prophylactic or second-line treatment [207, 208]. JAK inhibiting treatments begin also to emerge to cure SR patients. Ruxolitinib, a JAK1/2 inhibitor, has been reported as salvage therapy for SR-
GVHD thanks to its potent anti-inflammatory properties [209]. Itacitinib, a specific JAK1 inhibitor, has also shown potential efficiency in phase I trials as first-line treatment against aGVHD [192].

3. Lactate dehydrogenase elevating virus

Lactate Dehydrogenase-Elevating Virus (LDV), discovered in 1960 by Riley, is a murine arterivirus belonging to the order of Nidovirales [210, 211]. LDV is a spherical envelope virus with a positive sense single-stranded RNA genome 14kb long [212].

a. Infection

LDV is extremely mouse specific and the infection results in lifelong persistent viremia regardless of the mouse strain, age, sex or immune status. Many investigations suggest that LDV rapidly infects a macrophage subpopulation, which is said to be non-essential in immune response and is involved in scavenging extracellular lactate dehydrogenase [213]. Infected cell subpopulations reside mainly in the red pulp of the spleen with other macrophage subsets and monocytes, and have also been found in lymph nodes, skin, liver and testis [214]. In infected cells, virions are formed by vesicles budding into the cytoplasm, which merge with the plasma membrane and are then released. The virus replicates exponentially and causes rapid lysis of these cells indirectly resulting in high levels of plasma lactate dehydrogenase [215]. More specifically in experimental conditions, the virus is detectable at 12-14 hours after infection. The peak of viremia is at 24 hours and viral titers begin to decrease after 48 hours. Viremia then persists at a low chronic level that is dependent on the replenishment of new macrophage targets [216].
Although LDV specifically infects a subpopulation of macrophages, the LDV-receptor is still not known. It is known however that a molecule sensitive to trypsin is necessary for efficient infection in some strains of mice [217].

b. Effects on immune system

LDV is not usually a pathogenic virus, although it induces some immunomodulatory effects in the host. Early in infection, pro-inflammatory cytokines (IL-6, IL-12, IL-15 and IL-18) are transiently produced [218, 219]. Importantly, LDV induces IFN related genes. LDV RNA can bind directly to TLR7 expressed on pDCs and induces type 1 IFN production early in immune response (24h after infection) [220] that can modulate IFNγ production. This IFNγ is mainly secreted by NK cells and the production can drive global activation of macrophages and DCs [221].

LDV also affects T and B cells. Type I IFN produced by pDC due to TLR7 stimulation, broadly activate T and B lymphocytes [220]. In addition, LDV inhibits IL-4 production leading to a suppression of Th2 responses and favouring the Th1 response. This T helper differentiation leads to IgG2a production and suppression of IgG1 or IgE production [222]. In addition, N-glycans expressed on the major viral envelop protein (VP3) induce a non-specific activation of B-lymphocytes resulting in hypergammaglobulinemia switched to IgG2a and IgG2b isotype that depends also on the presence of functional T helper lymphocytes and IL-6 [223-225].

As mentioned before, LDV is not usually a pathogenic virus. However, certain quasispecies of LDV can induce polioencephalomyelitis in genetically susceptible mice [226]. Mice infected by LDV have a strong susceptibility to
septic shock induced by LPS. This effect is probably due to an imbalance in cytokine production in response to LPS in infected mice [222].

LDV can also have an effect on autoimmune diseases. For instance, it decreases the production of antibodies involved in severe immune complex glomerulonephritis reducing the incidence of mortality [227]. It exacerbates autoantibody-mediated hemolytic anemia [228] and thrombocytopenic purpura [229]. Nevertheless, LDV can suppress the development of experimental allergic encephalomyelitis [230].

Almost half a century ago, in 1969, Howard and Notkins reported that LDV infection prolongs skin allograft survival and inhibits spleen cell enlargement in a parent to F1 GVHD model [231].

4. R848/Resiquimod

R848 resiquimod is an imidazoquinoline that stimulates TLR7 in mouse and human and TLR8 only in human. TLR7 is an innate immune receptor localized intracellularly in the endosome and that binds viral single strand RNA [232, 233]. In human, TLR7 is expressed in several immune cell types, including pDCs, B cells, natural killer T cells (NKT), CD4 T cells and eosinophils [234-237] whereas, in mouse, it is mainly restricted to pDC and B cells [233, 238]. TLR8 is highly homologous to TLR7 and also binds single stranded viral RNA [239, 240] TLR8 is mainly expressed in human monocytes, myeloid DCs (mDCs), and neutrophils [241, 242]. Dependent on the context, TLR7/8 stimulation is able to activate the myeloid differentiation primary response 88 (MyD-88) pathway leading to pro-inflammatory cytokine production and IRF7 leading to type I IFN upregulation [240, 243].
Introduction

R848 was initially developed as an antiviral agent since it could replicate the protective effects of a virus [244]. R848 has shown an ability to activate mouse pDCs and CD8α+ cDCs in vitro resulting in IL-12p40 production and an increase of activation marker CD40 [245]. Similar results have been observed with human monocyte-derived dendritic cells in the Myd88 pathway [240, 246]. In addition, R848 is able to polarize T cells towards a Th1 phenotype [247]. In the mouse, adoptive transfer with invariant NKT cells stimulated with R848 in vitro alleviates allergic inflammation in a IFNγ dependent process [248]. R848-mediated Th1 response leads to an inhibition of Th2 cytokines involved in allergies. Therefore, several studies have highlighted the curative effect of R848 on these pathologies [249, 250].

On the other hand, stimulation of anti-tumor immunity by R848 was clearly illustrated in a mouse model of lymphoma. Daily systemic administration of R848 combined with radiotherapy led to a strong reduction of tumor development in a CD8 T cell dependent process [251]. A repeated cycle of R848 administration also seems to improve the efficacy of anti-cancer immune response against CT26 tumor [252].

However, the activity of R848 is not limited to stimulating Th1 cells. It also stimulates B cell proliferation and antibody production [253] as well as regulatory T cell-mediated TGF-β-dependent suppression of allergic asthma [254].

R848 induces a transient immune incompetence resulting from leukocyte trapping on epithelial cells in peripheral organs due to upregulation of ICAM-2 and P-selectin [255]. Daily administration of R848 to naive mice for one week leads to an immune activation with lymphoid system disruption, resembling HIV-mediated pathology [256].
5. Interleukin-27

Interleukin-27 (IL-27), belonging to the IL-6 and IL-12 family, is a cytokine formed by non-covalently linked p28 and Epstein-Barr virus-induced gene 3 product (EBI3) [257]. IL-27 binds to a heterodimeric receptor formed by the combination of WSX-1/IL-27Rα and gp130, which is expressed on all hematopoietic cells, vascular endothelium cells and keratinocytes [258]. IL-27 is known to be a pleiotropic cytokine. It is involved in the control of innate and adaptive immunity to infection. Produced by APCs, it is a pro-inflammatory cytokine that favors the development of T\(_{\text{h}1}\) CD4 T cells by inducing expression of IL-12Rβ2, which then enables IL-12 to stimulate IFNγ production [259, 260]. IL-27 also promotes CD8+T-cell maturation into fully differentiated cytolytic T lymphocytes and enhances granzyme B expression [261]. Its T\(_{\text{h}1}\) properties have already been reported in GVHD. Marillier and colleagues have demonstrated the essential role of IL-27 to mount a T\(_{\text{h}1}\) response and to induce disease in a parent to F1 aGVHD model [1]. In addition, through STAT-1 stimulation, which promotes T\(_{\text{h}1}\)-cell-specific transcription factor T-bet, IL-27 prevents CD4 T cells to develop a T\(_{\text{h}2}\) response [259, 262].

On the other hand, IL-27 is also known to negatively regulate the development of IL-17-producing T helper cells, leading to a decrease of inflammation in autoimmune diseases such as EAE [263-265]. This suppressive activity is probably mediated by its ability to induce IL-10 production [266]. Several studies have highlighted the immunosuppressive role of IL-27. Following infections, production of type I IFNs leads to upregulation of IL-27 and the subsequent promotion of IL-10 [267-269]. These findings are quite paradoxical and illustrate the pleiotropic function of IL-27.

Finally, it has been reported that IL-27 mediates complete regression of metastatic murine neuroblastoma tumors via CD8+ T cell activity [270]. This
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anti-tumor effect was also observed in human acute myeloid leukemia cells where it inhibits tumor expansion [271].
AIM OF THE STUDY
Aim of the study

In 1969, lactate dehydrogenase-elevating virus (LDV) was reported to prolong skin allograft survival in a parent to F1 non-irradiated GVHD model. However, no data were provided on the effect of the virus on final GVHD outcome and mechanistic analysis was of course limited by the available technology.

Therefore, the initial part of this work was to attempt to better analyze the effects of LDV in a parent to F1 non-conditioned (nc) GVHD model. The first objective was to characterize the GVHD protection. The second objective was to precisely identify which cells and cytokines were involved in this mechanism. The last objective was to determine if LDV effect is dependent of TLR7 activation since this virus activates TLR7 signaling.

The second part was to replace LDV by TLR7 agonist resiquimod (R848), an imidazoquinoline originally developed as an antiviral agent and an immune adjuvant. R848 was first tested in a parent to ncF1 GVHD model. As this was successful, the next objective was to identify mechanisms involved in this phenomenon. The last objective was to determine whether R848 treatment could still protect recipient mice in acute irradiated GHVD models and, if not, could it be complemented by anti-IL-27 blocking antibody?
RESULTS
Results

I. Mouse nidovirus LDV infection alleviates graft versus host disease and induces type I IFN-dependent inhibition of dendritic cells and allo-responsive T cells.

II. Blockade of interleukin-27 signaling reduces GVHD in mice by augmenting Treg reconstitution and stabilizing Foxp3 expression.

III. TLR7 ligand R848 prevents mouse GVHD and cooperates with anti-IL-27 mAb for maximal protection and Treg upregulation.
Results

Immunity, Inflammation and Disease

ORIGINAL RESEARCH

Mouse nidovirus LDV infection alleviates graft versus host disease and induces type I IFN-dependent inhibition of dendritic cells and allo-responsive T cells

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Keywords
DC, GVHD, nidovirus, type I IFN

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Funding information
This work was supported by the Belgian National Fund for Scientific Research (FNRS), the "Fondation Centre Le Canard" (FLC) (2010-166), the Joseph Maxim Fund and the Interuniversity Attraction Pole of the Belgian Federal Science Policy.

Received: 13 December 2016; Revised: 20 January 2017; Accepted: 8 February 2017
Final version published online: 4 April 2017.

Immunity, Inflammation and Disease
2017; 5(2): 200-213
doi: 10.1002/iid.157

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Abstract

Introduction: Viruses have developed multiple mechanisms to alter immune reactions. In 1968, it was reported that lactate dehydrogenase-elevating virus (LDV), a single stranded positive sense mouse nidovirus, delays skin allograft rejection and inhibits spleen alterations in graft versus host disease (GVHD). As the underlying mechanisms have remained unresolved and given the need for new therapies of this disease, we reassessed the effects of the virus on GVHD and tried to uncover its mode of action.

Methods: GVHD was induced by transfer of embryonic (E6) spleen cells to non-infected or LDV-infected B6D2F1 recipients. In vitro mixed-lymphocyte culture (MLC) reactions were used to test the effects of the virus on antigen-presenting cells (APC) and responder T cells.

Results: LDV infection resulted in a threefold increase in survival rate with reduced weight loss and liver inflammation but with the establishment of permanent chimerism that correlated with decreased interleukine (IL)-27 and interferon (IFN) γ plasma levels. Infected mice showed a transient elimination of splenic CD4+ and CD8+ conventional dendritic cells (cDCs) required for allogeneic CD4 and CD8 T cell responses in vitro. This drop of APC numbers was not observed with APCs derived from toll-like receptor (TLR)7-deficient mice. A second effect of the virus was a decreased T cell proliferation and IFNγ production during MLC without detectable changes in Foxp3+ regulatory T cell (Tregs) numbers. Both CD4 and responder T cell inhibition were type I IFN dependent. Although the suppressive effects were very transient, the GVHD inhibition was long-lasting.

Conclusion: A type I IFN-dependent suppression of DC and T cells just after donor spleen cell transplantation induces permanent chimerism and donor cell implantation in a parent to F1 spleen cell transplantation model. If this procedure can be extended to full allogeneic bone marrow transplantation, it could open new therapeutic perspectives for hematopoietic stem cell transplantation (HSCT).

Introduction

Hematopoietic stem cell transplantation (HSCT) is still the only curative treatment for severe malignant hematologic disorders but remains hampered by graft versus host disease (GVHD). This complication is fatal in approximately 15% of transplant recipients [1, 2]. GVHD also limits HSCT therapies in non-malignant hematopoietic disorders such...
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as Sickle cell disease [3], aplastic anemia [4], and immune deficiencies like AIDS [5].

GVHD occurs when donor T cells mount a strong immune response against the host after activation by minor and major histocompatibility antigens and cytokine storms induced by recipient conditioning [6]. Antigen presentation plays a major role in the initiation of this process [7] and its inhibition, for example by blockade of costimulation, has been explored as a potential therapeutic approach of human HSCT [8]. Similarly, in the mouse, inhibition of costimulation during antigen presentation was reported to limit disease [9]. However, questions remain regarding the type of antigen-presenting cells (APCs) critical for initiating the process. Host dendritic cells (DCs) are clearly potent GVHD initiators [10, 11] but non-hematopoietic recipient APCs may also induce alloreactive donor T lymphocyte activation and acute lethal GVHD [12]. At later time points, cross-presentation by donor APCs results in persistent pathogenic T cell stimulation [13].

Infectious agents through their pathogen-associated molecular patterns (PAMPs) and stimulation of innate pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs) or nod-like receptors (NLRs) are significantly implicated in GVHD development. A major source of pathogens and PAMPs is initially the gastrointestinal tract but, later, microbes translocate systemically due to damage of epithelial barriers after allo-HSCT [14]. This is the case for lipopolysaccharide (LPS), a component of Gram-negative bacteria, which is a potent TLR4 activator, contributing to severe GVHD via tumor necrosis factor-α (TNFα) secretion. The critical involvement of LPS-TLR4 was confirmed with an LPS antagonist that decreases GVHD severity [15, 16]. In humans, colonization of intestine by Candida spp leads to a severe GVHD as compared to uncolonized patients [17]. On the other hand, certain commensal bacteria such as Lactobacilli seem to play a beneficial role in mouse GVHD pathogenesis. Elimination of this species from the mouse flora before allo-HSCT aggravates GVHD whereas its reintroduction has the opposite effect [18]. Also, under certain conditions, TLR4 activation seems to have a beneficial role against the disease [19]. Together, these data show that environmental factors can both positively and negatively influence HSCT outcome.

In 1966, lactate dehydrogenase-elevating virus (LDV), a single stranded positive-sense RNA enveloped mouse nidovirus [20], was reported to prolong skin allograft survival and to inhibit spleen size changes in a parent to F1 non-irradiated GVHD model [21]. However, no data were provided on the effect of the virus on final GVHD outcome and mechanistic analysis was of course limited by the available technology. To the best of our knowledge, no attempt to better characterize the effects of LDV in GVHD has been reported since.

Results

LV infection prevents acute B6 to B6D2F1 GVHD mortality and morbidity

We tested the effect of LDV infection on acute GVHD (aGVHD) induced in B6D2F1 recipients of B6 spleen cells. Infection of recipient mice with LDV 24 h before B6 cell transfer conferred significant protection against disease. In pooled data of five experiments involving a total of 28 control and 27 infected mice (Fig. 1A), mortality was significantly decreased after infection, dropping from 75% in control to 25% in LDV-infected animals. Moreover, weight loss, a marker of morbidity in mouse aGVHD, was completely suppressed in the infected survivors (Fig. 1B).

LV infection inhibits IFNγ and IL-27 production and prevents liver and spleen damage

According to previous work [22], in the B6 to B6D2F1 model of GVHD, IFNγ and IL-27 are good markers of acute GVHD that peak shortly after allogeneic cell transfer. We therefore measured these cytokines in the serum six and 10 days after cell transfer. LDV infection significantly decreased IFNγ and IL-27p28 levels in the serum compared to control GVHD mice (Fig. 2A and B).

Liver tissue damage is an important marker of acute GVHD. Histological analysis was therefore performed on liver sections between 14 and 18 days after B6 spleen cell transfer to B6D2F1 mice (depending on disease severity). Large lesions were found in the livers of control mice that received B6 spleen cells but not in LDV-infected mice. However, LDV infection did not prevent mononuclear cell
Results

Viral impairment of GVHD

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Figure 1. LDV infection protects mice against aGVHD. B6D2F1 recipient mice were infected or not with LDV 24 h before transfer of 60 x 10^6 B6 splenocytes. (A) Mice were monitored for mortality. Data are from five pooled experiments with a total of 28 control and 27 infected mice (P < 0.01, log-rank Mantel-Cox test). (B) Mice were monitored for weight loss. Data are mean ± SEM (n = 5 mice per group) of one experiment and representative of three (P < 0.05 by Anova-Bonferroni post hoc). Infiltration as illustrated in the representative micrograph shown in Figure 2C.

LDV infection allows establishment of donor-host splenic chimerism

To determine whether B6 splenocytes were able to engraft in LDV-infected mice, we labeled B6D2F1 spleen cells 14 days after B6 spleen cell transfer with anti-MHC class-I haplotype antibodies (anti-H-2D^b/D^b for B6 and anti-H-2D^b/D^b for B6D2F1) (Fig. 3A). Flow cytometry analysis indicated that donor cells were present in the spleen of infected mice and that their numbers increased as compared to non-infected recipients but the difference did not reach statistical significance (8.8 ± 3.6 x 10^6 in controls and 21.8 ± 8.5 x 10^6 in LDV mice [P > 0.05]) (Fig. 3B). A similar trend was seen for CD4 and CD8 T cells but B cells were barely detectable (Fig. 3C–E). On the other hand, host spleen cell survival was dramatically increased in infected mice (LDV: 2.7 ± 4.1 x 10^9 vs. control GVHD: 2.7 ± 0.7 x 10^9, P < 0.01 as compared to 60.7 ± 6.8 x 10^5 in normal B6D2F1 mice). In fact, the destruction of host CD4 and CD8 T cells was completely inhibited by LDV while that of host B cells was only partially but significantly diminished (Fig. 3C–E). Thus, LDV did not at all impair donor T cell implantation but inhibited host B and T cell depletion.

LDV infection inhibits both CD4 and CD8 T cell stimulation

LDV has been reported to impair MHC class II-mediated protein antigen presentation to in vivo primed T lymphocytes [23]. To determine whether this mechanism also operates in allogeneic reactions, we tested the influence of the virus on allogeneic mixed-lymphocyte-culture (MLC). B6D2F1 (H-2D^b/D^b) mice were infected or not with LDV 72 h before co-culture of their adherent spleen cells with normal B6 responder splenocytes (H-2D^b). Both proliferation and IFNγ production were strongly inhibited if stimulator cells were collected 72 h after LDV infection (Fig. 4A and B). Similar observations were made in other fully allogeneic reactions such as FVB (H-2b) > 129/Sv (H-2b) (Fig. 4C), CBA (H-2k) > 129/Sv, or B6 > BALB/c (H-2d) (data not shown). Of note, any action on the responder cells of traces of the virus remaining in the APC preparation is ruled out by the short survival time of the virus in vitro [24].

The kinetics of LDV-mediated suppression was tested in the FVB > 129/Sv combination. Abrogation of the response was maximal if stimulator cells were prepared from spleens collected one or three days after infection. Both proliferation (and IFNγ production, data not shown) started to recover after six days and returned to normal after 12 days (Fig. 4C).

The suppression of allogeneic reactions by LDV described in the allogeneic MLC experiments suggests that both class I and II antigenic reactions were suppressed. To directly address this question, adherent spleen cells from 129/Sv mice (H-2b) were incubated with purified CD4^+ or CD8^+ T cells (anti-MHC class II Ab was added to inhibit background MHC class II reactions in CD8 experiments) from FVB (H-2b) spleens. Total splenocytes, purified CD4^+ and CD8^+ responder cells incubated with APCs from LDV-infected mice all had significantly reduced proliferation compared to responder cells incubated with APCs from non-infected mice (Fig. 4D). Together, the data indicate that LDV infection transiently affects splenocyte APCs that become unable to stimulate allogeneic CD4^+ and CD8^+ T cells.

LDV inhibits sDC stimulatory activity of allogeneic spleen cells in vitro

To analyze the mechanisms underlying the suppressive effects of LDV on allogeneic Ag stimulation, we first identified the allogeneic Ag presenting spleen cells responsible for T cell activation in vitro. Initial analysis showed that CD11c^+ cells but not CD11b^+CD11c^+ cells...
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Figure 2. LDV infection lowers plasma inflammatory cytokine levels and abrogates liver lesions. B6D2F1 mice were infected or not with LDV 24 h before transfer of 6 x 10⁶ B6 spleen cells. (A) IFN-γ and IL-12p40 were measured by ELISA in plasma prepared from mice killed on days 6 and 10. Data are means ± SEM (n = 3 mice per group for day 6 and n = 5 mice per group for day 10) and representative of two independent experiments (**P < 0.01, ***P < 0.001 by 2-way ANOVA and Bonferroni post-test). (C) Liver sections were prepared from normal B6D2F1 or control or LDV mice at day 16 of GVHD. Mononuclear inflammatory cells present in portal tracts (green arrow) and large-dilated sinusoidal spaces (red arrow) are indicated. Scale bars in upper and lower panels represent 200 and 50 μm, respectively. Slides are representative of two independent experiments with five mice per group.

(mainly macrophages) stimulated allogeneic T lymphocytes (Supplementary Fig. S1A). Further characterization of the allogeneic stimulating APC population performed by FACS sorting (Supplementary Fig. S1B) indicated that CD11c⁺,MHC II⁺,B220⁻,CD11b⁺ and CD11c⁺,MHC II⁺,B220⁻,CD8α⁺ conventional DCs (cDCs) but not CD11c⁺,MHC II⁺,B220⁻,pDCCA⁺ pDCs were effective stimulators of allogeneic responder spleen cells (Fig. 5A and C). Quantification of splenic CD11b⁺ and CD8α⁺ cDCs from control or LDV-infected mice showed that both were severely diminished after infection (CD11b⁺: 4.3 ± 0.3 vs. 0.33 ± 0.03 x 10⁶ and CD8α⁺: 5.3 ± 0.7 vs. 0.58 ± 0.09 x 10⁶) (Fig. 5C and D).

To evaluate whether this cell loss was due to lytic infection or to soluble factors like type I IFNs, which have been reported to be strongly induced early after infection [21], we repeated the infection in 129/Sv H2b wild type or 129/Sv-IFNAR⁻/⁻ mice and observed that, in IFNAR⁻/⁻ mice,
LDV did neither decrease the number of CD11b<sup>+</sup> and CD8α<sup>+</sup> cDCs (Fig. 5C and D) nor their ability to function as APC in MLC reactions using FVB (H<sup>2</sup><sup>b</sup>) responder spleen cells (Fig. 5E). Of note, 129/Sv-IFNAR<sup>−/−</sup> mice were not resistant to LDV infection as their lactate dehydrogenase serum titer was similar to that of control 129/Sv-infected mice (data not shown).

LDV was reported to induce TLR7 activation [26]. We verified that TLR7 was required for the suppression of allo-antigen stimulation by incubating LDV-infected or control spleen cells from wild type or TLR7<sup>−/−</sup> B6 mice with BALT/c (H<sup>2</sup><sup>b</sup>) responder spleen cells. Although the LDV suppressive effect on B6 APCs was not as strong as in 129/Sv APCs, we observed that the APC function of TLR7<sup>−/−</sup> was contrary to that of wild-type mice, was not affected by LDV (Fig. 5F). In addition, cDC numbers were not decreased in TLR7<sup>−/−</sup> B6 spleens compared to LDV-infected WT B6 (Supplementary Fig. S2). These results suggest that only...
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conventional DCs are able to activate alloimmune T cell response in vitro and, following LDV-infection, splenic cDCs are eliminated. This effect is type I IFN and TLR7 dependent.

LDV infection decreases the reactivity of alloimmune responder T cells

It is well established that if the alloimmune T cells are removed from donor graft, GVHD will not be induced [27, 28]. Therefore we investigated the influence of LDV on the responder component of GVHD. B6 mice were infected at several time points before testing the response of their spleen cells to non-infected B6D2F1 APCs in vitro. These experiments showed a significant decrease in proliferation and IFNγ production that was, however, only transient (at day 3 not at day 1 or 6) (Fig. 6A and B).

To formally prove that LDV had actually modified the responder T cells, we purified splenic CD5+ T cells (to include both CD4 and CD8 T cells) from control or LDV-infected B6 mice by positive selection and tested their allo-reactivity in MLC. If the T cells were collected three days after LDV infection, both proliferation and IFNγ production were strongly inhibited (Fig. 6C and D).
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Figure 5. LDV infection affects conventional dendritic cell stimulation of allo-recipient MNC in a type IIFN- and TLR7-dependent process. (A) and (B) FVB cD3 spleen cells were incubated without APC or with CD11b+ cDCs, CD8a+ cDCs or pDCs purified by MACS beads and FACS sorting from normal 129/Sv mice. After 48 h, proliferation was measured by 3H-thymidine incorporation. (A) Representative experiment (mean ± SD of experimental triplicates). (B) Fold induction compared to splenocytes. Data are pooled from three independent experiments (*P < 0.05, Kruskal–Wallis test with Dunn’s Multiple Comparison Test). (C) and (D) Spleen cells from naïve and three-day LDV-infected or un-infected 129/Sv or 129IFNAR-1ΔΔ mice were labeled with anti-CD11c, anti-CD8a, anti-CD14, and anti-CD86 Abs and analyzed by FACS. The total number of splenic CD11c+ cDCs and CD8a+ cDCs are represented by dot plots. Data are means ± SEM (n = 4 mice per group) and representative of four independent experiments (*P < 0.05, **P < 0.01, Kruskal–Wallis test with Dunn’s Multiple Comparison Test). (E) Type IIFN signaling is required for LDV suppressive activity. Splenic APC from one-day LDV-infected and naïve 129/Sv WT or IFNAR-1ΔΔ mice were incubated with normal FVB spleen cells. The proliferation was measured by 3H-thymidine incorporation. Data are means ± SEM (n = 4 mice per group) and representative of three independent experiments (*P < 0.05, Kruskal–Wallis test with Dunn’s Multiple Comparison Test). (F) TLR7 activation is required for LDV suppressive activity. APC from one day LDV-infected and naïve 129/Sv WT or 86 TLR7ΔΔ mice were isolated from the spleens and incubated with normal FVB spleen cells. The proliferation was measured by 3H-thymidine incorporation. Data are means ± SEM of two pooled experiments (n = 5–12 mice per group) representative of three experiments (*P < 0.05, Kruskal–Wallis test with Dunn’s Multiple Comparison Test).
Results

LDV impairs B6 response to B6D2F1 APC

A

Thymidine CPM x 10^3

Control 1 2 3

BD2F1 APCs  No APC

B

IFN-γ (pg/mL)

Days after LDV infection

Control 1 3 6

C

Thymidine CPM x 10^3

Control LDV

B6 CD5 responder cells

D

IFN-γ (pg/mL)

Control LDV

B6 CD5 responder cells

Figure 6. LDV causes transient hyporesponsiveness in CD5+ spleen cells three days after infection. Infection kinetics. B6 spleen cells were recovered at various time points after LDV infection and incubated with B6D2F1 irradiated adherent stimulating cells for 48 h. (A) Cells were pulsed with 3H-thymidine (or 18 h) to determine proliferation. (B) After 12 h, IFN-γ production was determined by ELISA. Data are means ± SEM (n = 5 mice per group) and representative of three independent experiments (*P < 0.05, 2 way ANOVA and Bonferroni post-tests). (C) and (D) The CD5+ B6 cell reactivity is affected after LDV infection. B6 responder spleen cells were recovered three days after LDV infection and CD5+ cells were purified by MACS before incubation with B6D2F1 adherent stimulating cells for 48 h. After 72 h, (C) proliferation and (D) IFN-γ production were determined. Data are means ± SEM of two pooled experiments (n = 6 mice per group) representative of three experiments (*P < 0.01, **P < 0.001, Mann-Whitney U-test).

To evaluate the involvement of regulatory T cells (Tregs) in the LDV suppressive process, we analyzed the number of Tregs in spleens, of normal and LDV-infected B6 mice at various time points using Fox3 staining and FACS analysis. No change in percentage or absolute number was observed in Fox3+ CD4+ T cells from one to six days after infection (Fig. 7A and B).

To test the implication of type I IFN signaling in the T cell inhibition, we infected 129/Sv or 129/Sv-IFNAR-1−/− mice and compared the proliferation of their spleen cells to that of controls in MLC with irradiated allogeneic adherent B6D2F1 spleen cells. This experiment confirmed the inhibitory effect of LDV on the allogeneic responder cells in a different strain combination and showed that, in 129/Sv-IFNAR-1−/− responder cells, a slight but non-significant inhibition was observed suggesting that type I IFN plays a major role in T cell inhibition by LDV but that part of the inhibition could be IFNAR-1 independent (Fig. 7C).

Finally, given the critical role of IFNAR-1 in the effect of LDV on cDC suppression and T cell allo-responsiveness, it was of interest to evaluate the presence of type I IFNs in infected and control GVHD mice at different time points. Figure 7D shows that type I IFN production occurred in a sharp peak 18h after infection in accordance with previously reported data [25]. This fits well with the very transient nature of DC and T cell alterations by LDV. Of note, type I IFN seems to be induced only by LDV infection and not by GVHD since one day after donor cell transplantation type I IFN was not detectable anymore. This implies that the donor cells were only shortly exposed to an IFN-rich environment but this was sufficient to impair their allogeneic response.
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Figure 7. The LDV suppressive effect on T cells is not related to a change in Treg number but is IFNAR dependent. (A and B) B6 spleen cells from LDV-infected and non-infected mice were recovered and stained with anti-TCRβ, anti-CD4, anti-FoxP3, and IFNAR1-APC to determine the total number of Treg cells by FACS. Data are means ± SEM (n = 5 mice per group) and representative of two independent experiments. (A) Representative density plots with the percentage of each gated population. (B) The total number of FoxP3+CD4+ TCRβ+ Treg cells from spleen are represented by scatter plots. (C) The suppression of responder cells is type I IFN dependent. Responder spleen cells from three day LDV-infected and non-infected 129/Sv WT (n = 6) or 129/Sv/IFNAR-1−/− (n = 6) mice were recovered and cocultured with normal B6 D2F1 APCs. Cells were pulsed with 3H-thymidine for 18 h to determine proliferation. Data are means ± SEM and representative of three independent experiments (***P < 0.001, Kruskal-Wallis test with Dunn's Multiple Comparison Test). (D) B6 D2F1 mice were infected or not with LDV at day 0. After 24 h, 60 x 10⁸ B6 spleen cells were injected ip to induce GVHD. Type I IFN activity was measured in plasma from mice bled at several time points and was quantified, as described previously, by a standard cytopathic effect reduction assay performed in 96-well plates on B95.8/FTS cells infected with Mongo virus [46]. The detection limit in plasma was 1375 units/ml (corresponding to 10 pg/ml of reference recombinant IFNa). Data are means ± SEM (n = 4 mice per group).
Discussion

It has been suggested that viral infections may in some circumstances predispose recipients of HSCT to severe GVHD [29]. However, increasing evidence indicates that infectious agents may also prevent this adverse reaction through their interaction with innate receptors [30, 31]. We used LDV as a tool to modulate the immune microenvironment during acute GVHD in a parent to non-conditioned F1 recipient model. We selected this model as an immunologic tool to avoid the massive inflammation induced by recipient irradiation so that the effect of the virus, if any, would be easier to analyze. This relatively mild GVHD, which nevertheless killed 50% of the recipients, was partially impaired by the infection, thus further extending the suppressive effects of this virus previously reported in various auto-immune diseases [32-34].

The protection conferred by LDV correlated with a short (±one week) inhibition of both allo-Ag presentation by DC and donor T cell responsiveness. Allogeneic T cell stimulation by spleen cells of LDV-infected wild type, but not TLR7-deficient, mice was severely compromised after infection which coincided with selective elimination of CD11b+ and CD8α+ cDCs from the spleen. LDV infection did not prevent donor cell implantation but inhibited weight loss, host lymphocyte destruction, liver damage as well as IFNγ and IL-27 production, two cytokines contributing to the pathology of parent to F1 GVHD [32, 35].

Our results confirm and expand data reported by Isakov who observed that LDV impaired presentation of a protein Ag by peritoneal macrophages without affecting their phagocytic activity [23]. In retrospect, these peritoneal cells were probably DC because we confirmed these observation with ovalbumin presentation and found that DC, not macrophages, were the main antigen presenting cells in this system (Guigashe, unpublished observations). We here extended these results to allogeneic responses and identified CD11b+ and CD8α+ cDCs as the main initiators of these responses contrary to macrophages that were very poor APCs in MLC reactions. Of note, LDV also induced partial disappearance of CD11c+ MHCII+ B220- pDCs but these cells were unable to induce allogeneic T cell responses in vitro, in agreement with published data [36]. Together, these results suggest that the impaired antigenic stimulation during LDV infection results from a selective suppression of cDC function providing further evidence for the critical role of these cells in GVHD, as originally reported by Shlomich et al. [37]. Of note, although the number of cDC completely normalized two weeks after infection, donor T cells persisted without inducing a delayed GVHD reaction. Moreover, LDV infection performed one week after B6 spleen cell transplantation no longer impaired GVHD [38] further supporting the action of the virus on the initiating steps of the response.

The suppressive effect of LDV on APCs was TLR7 dependent in agreement with the reported activation of TLR7 by the virus [38] and the impairment of DC differentiation and maturation by TLR7 agonist Resiquimod [39]. Given that TLR7 agonist increases IFN-β production [40], and that APCs from IFNAR-1−/− mice were not affected by LDV, the TLR7-type I IFN pathway seems to play a central role in the suppressive effect of LDV on allo-Ag presentation. Of note, type I IFN activity in plasma peaked at 18 h after infection which fits well with the timing of DC cell depletion.

The above results suggested that inhibition of APCs could explain the protective effect of LDV in GVHD. However, severe depletion of host cDCs does not prevent GVHD as reported in CD11c-DTR transgenic recipients where host cDCs were depleted by diptheria toxin [41]. Based on these observations, the protective effect of LDV was probably not just due to suppression of cDCs. In fact, CD4+ T cells from LDV-infected donor mice also showed poor proliferation and IFNγ production in MLR. This inhibition was also partly dependent on type I IFN, in line with the reported exacerbation of GVHD in IFNAR-1−/− B6 recipients of BALB/c CD4 T cells and by the inhibition of allogeneic donor T cell responses by IFNα [42]. However, other mechanisms may still be implicated as T cell inhibition by LDV was not completely abrogated in IFNAR-1−/− mice.

Finally, we confirmed the early and brief presence of type I IFN activity in LDV-infected mice described earlier [25] and showed that it was not prolonged by GVHD induction. In view of the requirement of IFNAR-1 for LDV suppressive effects in allogeneic reactions, it follows that very short exposure to type I IFNs could have profound effects on GVHD outcome.

An explanation of the protective effects of LDV infection on GVHD would thus be that massive viral replication, which peaks within the first 20 h after infection, induces strong viral RNA-mediated activation of TLR7 which in turn elicits a strong but very transient production of type 1 IFN which affects antigen presentation by cDC and partly T cell alloresponsiveness. These changes last only a few days, suggesting that critical events occur shortly after HSCT and that their alteration can have long lasting consequences on GVHD outcomes. This observation could open new perspectives for the design of GVHD prevention protocols.

Materials and Methods

Mice

Most mice were bred under SPF conditions at the animal facility of the Ludwig Institute Brussels Branch under the

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Direction of Guyl Warnier and Gilles Gaudray (DVM). Experimental protocols and animal handling were approved by the ethical committee of the Medical Faculty of the Université de Louvain (accrualation n°: 2011/UCL/MD/014). IFN-α/βR−/− 129/Sv mice (IFNAR−/−) were a gift of Dr. M. Aguet (43). TLR7−/− (44) were provided by and maintained in the animal facility of the CNRS Orleans, LDV, Riley strain, originally obtained from the ATCC, was maintained in our laboratory by passage in NMRI mice. Infection was performed by intra-peritoneal injection of a 2 × 10^5 infectious dose 50 (ID50) of LDV.

Induction of GVHD

GVHD was induced by i.p. injection of 50–70 million C57Bl/6 (B6) spleen cells in B6xDBA/2 F1 (B6D2F1) recipients one day after LDV infection. All experiments were performed on adult mice with a weight of 20 g. Mice were monitored for survival and weight loss every other day. Mice were bled on the indicated days to monitor chimerism and serum cytokines and at the experimental endpoint, euthanized for spleen and liver analyses.

In vitro culture

MLC was carried out by incubating 1.25 × 10^6 responder spleen cells/ml with an equal number of irradiated (30 Gy from a 137 Cs source) adherent spleen cells. In some cases, responders were CD4+, CD8+, and CD19+ cells purified from spleens and seeded at a density of 0.25 × 10^6 cells/ml. Adherent cells were obtained by coating 1 × 10^6 splenocytes in a 96-well flat bottom microtiter plate for 1.5 h and removing non-adherent cells by washing the microplate twice with PBS (37°C). DC subpopulations were cultured at 10^5 cells per well. Proliferation was measured after two days by incubation with 3H-thymidine at 1 μCi (0.037 MBq)/well for a further 18 h. 3H-thymidine incorporation was measured using a scintillation counter (Packard Microplate Scintillation Counter). MHC II activity was blocked in vitro using anti-MHC II LEAF anti-mouse I-A/I-E clone M5/I14.15.2 (Biolegend, San Diego, CA) at 5 μg/ml.

Flow cytometry and cell sorting

To determine chimerism, splenocytes were stained with anti-H-2D^b-PE (clone: 34-2-12) and anti-H-2D^k-PE (clone: K195) (all from Biolegend). Treg cells were determined in the spleens according to manufacturer’s instructions using a kit from eBioscience (San Diego, CA). Spleen cell were also characterized using anti-CD11b (clone: M170), anti-CD11c (clone: N418), anti-B220 (clone: RA3-64.2), anti-I-A/I-E (clone: M5/I14.15.2), anti-CD4 (clone: RM4-5 and GK1.5), and anti-pDC (clone: 100712), anti-TCR-β (clone: H57-597), all from Biolegend, and a viability marker (LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit, Life Technologies, Eugene, OR). For MACS cell purification, we used anti-CD11c (cat. 130-052-001), anti-CD4 (cat. 130-049-201), and anti-CD8 (cat. 136-049-301) microbeads (Miltenyi Biotec Bergisch Gladbach, Germany). For the in vitro functional assays, DC subpopulations were purified from spleens by MACS. CD11c cells were then sorted by FACS (BD FACS Aria III) using APC/Cy7-labeled anti-B220, PercP-labeled anti-I-A/I-E, APC-labeled anti-pDC, PE-labeled anti-CD11b and PE/Cy7-CD8α. We obtained 90.9 ± 1.55% purity for CD8α+DCs, 95.1 ± 1.5% for CD11b + DCs and 94.0 ± 0.3% for pDCs. All cells were acquired using a FACS LS R Fortessa according to BD bioscience protocols and analyzed by Flowjo software version 9.8.1.

Cytokine measurements

Cytokine production was measured in cell culture supernatants and serum. ELISA specific for murine IFN-γ (R&D Systems) was performed, according to manufacturer’s instructions. IL-27p28 was measured using mAbs generated in our laboratory as previously described (45). In all ELISAS, biotinylated detection Abs were used followed by avidin-HRP (Biolegend). All absorbance reads are made at 450 nm, using a 96-well plate spectrophotometer (VERSAMax, Molecular Devide).

Type I IFN bioassay

Type I IFN activity was measured by a cytopathic effect reduction assay as described in Figure 7D (46).

Statistical analysis

Statistical analysis was performed with instat data analyzer and Prism 5 (Graphpad Software, La Jolla, CA) using non-parametric tests (Kruskal-Wallis or Mann-Whitney), parametric test (Bonferroni’s multicomparison), and Log-rank Test for survival curve.

Acknowledgments

This work was supported by the Belgian National Fund for Scientific Research (FRS), the “Fondation Contre le Cancer” (FCC) (2010-165), the Joseph Maisin Fund and the Interuniversity Attraction Pole of the Belgian Federal Science Policy, M.G. is FRS-FRIA PhD Fellow at the Université Catholique de Louvain. B.G.M. is a Haas-Teichman fellow of the de Duve Institute and Télérie funded post-doctoral fellow (F5/202). I.P.C is FRS research director. The technical help of Pamela Choua is gratefully acknowledged.
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Author Contributions

MG and RGM contributed equally to the design and performance of experiments, analysed data, and wrote the paper. CU contributed to the development of the project and critically read the paper. ND designed and performed FACS sorting experiments, AS and TM performed experiments and analyzed data; JR suggested the use of C57BL/6 TLR7-Kos and RGM, IJS, and JPC conceived the research project, designed experiments, and wrote the paper.

Conflict of Interest

The authors declare no financial or commercial conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web-site.

Figure S1. Only conventional DCs are able to activate allogeneic T cell response in vitro.

Figure S2. LDV infection affects cDCs in a TLR7-dependent process.
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TRANSPANTATION

Blockade of interleukin-27 signaling reduces GVHD in mice by augmenting Treg reconstitution and stabilizing Foxp3 expression


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Key Points
- Blockade of IL-27 signaling mitigates the severity of GVHD by recalibrating the effector and regulatory arms of the immune system.
- Inhibition of IL-27 augments the reconstitution of CD4+ and CD8+ regulatory T cells and increases the stability of Foxp3 expression.

Reestablishment of competent regulatory pathways has emerged as a strategy to reduce the severity of graft-versus-host disease (GVHD), and recalibrate the effector and regulatory arms of the immune system. However, clinically feasible, cost-effective strategies that do not require extensive ex vivo cellular manipulation have remained elusive. In the current study, we demonstrate that inhibition of the interleukin-27p28 (IL-27p28) signaling pathway through antibody blockade or genetic ablation prevented lethal GVHD in multiple murine transplant models. Moreover, protection from GVHD was attributable to augmented global reconstitution of CD4+ natural regulatory T cells (nTregs), CD4+ induced Tregs (iTregs), and CD8+ Tregs, and was more potent than temporally concordant blockade of IL-6 signaling. Inhibition of IL-27p28 also enhanced the suppressive capacity of adoptively transferred CD4+ nTregs by increasing the stability of Foxp3 expression. Notably, blockade of IL-27p28 signaling reduced T cell-derived IL-10 production in conventional T cells; however, there was no corresponding effect in CD4+ or CD8+ Tregs, indicating that IL-27 inhibition had differential effects on IL-10 production and preserved a mechanistic pathway by which Tregs are known to suppress GVHD. Targeting of IL-27 therefore represents a novel strategy for the in vivo expansion of Tregs and subsequent prevention of GVHD without the requirement for ex vivo cellular manipulation, and provides additional support for the critical proinflammatory role that members of the IL-6 and IL-12 cytokine families play in GVHD biology.

Introduction

Graft-versus-host disease (GVHD) is characterized by the increased production of inflammatory cytokines, activation and expansion of alloreactive donor T cells, and the failure of existing regulatory mechanisms to counterbalance this proinflammatory milieu. The latter, in particular, has been a major focus of inquiry given that GVHD is characterized by impaired reconstitution of regulatory T cells (Tregs) which contributes substantially to the pathophysiology of this disease. That observation has been the impetus for strategies directed at the reestablishment of an effective Treg network by the adoptive transfer of ex vivo-expanded Tregs. Although these studies have demonstrated feasibility, there have been no controlled studies demonstrating efficacy, and the technology necessary for this approach is not widely available to all transplant centers. Thus, alternative strategies designed to facilitate the in vivo expansion of existing Treg populations by modulating the inflammatory cytokine milieu via antibody blockade or exogenous cytokine administration have intrinsic merit given the potential broader clinical availability of these approaches.

Interleukin-6 (IL-6), along with other IL-6 cytokine superfamily members such as IL-23, has been shown to have an important proinflammatory role in GVHD in both preclinical murine models and recent clinical studies. IL-27, another member of the IL-6 cytokine family, is a heterodimeric cytokine that is composed of p28 and Epstein-Barr-induced gene 3 (EBI3) subunits and signals through a heterodimeric receptor composed of WSRF-1 and gp130 which is part of the IL-6 signaling complex. Like IL-23, IL-27 is secreted by activated antigen-presenting cells (APCs) such as macrophages, monocytes, and dendritic cells and signals through Stat3. The IL-27R is highly expressed on effector memory CD4+ and CD8+ T cells,) and ligand of the receptor leads to Stat1 and Stat3 activation. Although initially thought to have proinflammatory effects, recent studies have uncovered an immunoregulatory role for IL-27 which has been derived from data showing that IL-27 suppresses retinoic-acid-related orphan receptor y (RORyt) T helper 17 (T(17)) development and increases T-cell (CD8+) production of IL-10.
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Figure 1. Transplantation with L.275p28<sup>−/−</sup> marrow grafts exacerbates GvHD. (A) Lethally irradiated (900 cGy) Balb/c mice were transplanted with BALB/c BM cells, n = 5-6 per time point, or together with BALB/c spleen cells (adjusted to yield an αT cell dose of 0.7 × 10<sup>6</sup>) (L, n = 5-11 per time point). Normal nontransplanted Balb/c mice served as controls (M, n = 6). Plasma IL-27p28 levels are depicted at each time point in individual animals. (B) IL-27 and IL-27R messenger RNA expression in the liver, lung, and colon of...
Notably, IL-27 has also been shown to affect Treg biology, although whether IL-27 inhibits or enhances Treg expansion remains controversial and appears to be dependent, in part, upon the experimental conditions. The goal of the current report therefore was to determine whether IL-27 exerted proinflammatory or immunosuppressive effects during GVHD, and to examine specifically the effect of IL-27 on the reconstitution of the Treg compartment under these inflammatory conditions.

**Results**

Transplantation with marrow grafts from IL-27p28−/− mice exacerbates GVHD lethality

IL-27 is a member of the IL-6 cytokine superfamily which includes IL-12 and IL-23. However, in contrast to these cytokines, which have predominantly inflammatory roles, IL-27 has both inflammatory and immunoregulatory effects in nontransplant models. 1,2,5,24-25 To define the role of IL-27 in GVHD, we first observed that plasma IL-27 levels were significantly increased in GVHD animals peaking at day 6 (Figure 1A), and that IL-27 and IL-27R messenger RNA levels were augmented in the liver, lung, and colon compared with bone marrow (BM) control animals (Figure 1B). Because IL-27 is secreted by APCs25 and has effects on T cells,2,5,26 both donor and recipient-derived IL-27 have the potential to modulate GVHD severity. We observed that transplantation with IL-27p28−/− donor grafts exacerbated GVHD mortality (Figure 1C) and accelerated weight loss (Figure 1D) compared with animals that received wild-type grafts. Histological analysis revealed that there was increased pathologic damage in the liver and lungs of these animals (Figure 1E). A characteristic in both liver and lung was the finding of increased numbers of inflammatory infiltrates in periportal and perivascular areas, respectively (Figure 1F). There was also an increased number of donor-derived CD4+ and CD8+ T cells in liver, lung, and colon, as well as a significant increase in CD4+ and CD8+ interferon-γ-positive (IFN-γ+) cells in the liver and lung of recipients of IL-27p28−/− marrow grafts (Figure 1G-H). Conversely, there was no difference in the absolute number of Tregs in the spleen, compared with IL-10−/− recipients or the parental strain (Figure 1I).
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Results

CD8⁺ T cells from IL-27⁺⁺ mice are inherently biased toward a proinflammatory phenotype which exacerbates GVHD

This discordance in results was unexpected and led us to consider whether there were baseline alterations in transgenic T cells from IL-27⁺⁺ animals that would cause these cells toward a more inflammatory phenotype. To determine whether this premise was correct, we performed phenotypic characterization and functional assessment on T cells from both wild-type and IL-27⁺⁺ mice. We observed a significant increase in the percentage of central memory CD69⁺, but not CD28⁺, T cells in the spleens of IL-27⁺⁺ mice and a corresponding reduction in the frequency of naive CD8⁺ T cells (Figure 3A-B).

Phenotypic examination of CD8⁺Foxp3⁺ T cells revealed no difference in expression of CD25, CD44, CD62L, CTLA4, CD103, glucocorticoid-induced tumor necrosis factor (GIFT), or CD27 (Figure 3C). In functional studies, there was no difference in proliferation when T cells from IL-27⁺⁺ mice were cultured in the presence of allogeneic APCs (Figure 3D). However, when splenic T cells were stimulated in vitro with phorbol 12-myristate 13-acetate (PMA)/ionomycin, we observed an increased percentage of CD8⁺⁺, but not CD8⁺, T cells (data not shown), which produced IFN-γ from IL-27⁺⁺ mice (Figure 3E). There was no difference in the percentage of either CD4⁺ or CD8⁺ T cells that expressed other inflammatory cytokines such as TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and an actual decrease in IL-2, after similar polyclonal activation (supplemental Figure 5A). Culture of CD4⁺ and CD8⁺ T cells under Th1 or Th2 polarizing conditions also revealed a significant increase in the percentage of CD8⁺⁺ IFN-γ⁺, but not CD8⁺ TNF-α⁺, T cells from IL-27⁺⁺ animals (Figure 3F). Th1-polarized CD8⁺ T cells from IL-27⁺⁺ animals also had increased expression of T-bet (supplemental Figure 5B). To determine whether CD8⁺⁻ T cells from IL-27⁺⁺ animals could exacerbate GVHD, we performed T-cell subset transfer experiments in which animals were reconstituted with CD8⁺⁺ and/or CD4⁺ T cells from either wild-type or IL-27⁺⁺ mice. Mice that were transfused with wild-type CD4⁺ and IL-27⁺⁺ CD8⁺ T cells had significantly worse survival compared with animals reconstituted with wild-type CD4⁺ and CD8⁺ T cells (Figure 3G). In contrast, transplantation with IL-27⁺⁺ CD4⁺ and wild-type CD8⁺ T cells did not increase GVHD severity relative to mice reconstituted with wild-type CD4⁺ and CD8⁺ T cells. Collectively, these results were evidence that the lack of endogenous IL-27 resulted in intrinsic immune dysregulation in CD8⁺ T cells which augmented their ability to exacerbate GVHD.

Transplantation with IL-27⁺⁺ marrow grafts reduces GVHD severity and accumulation of proinflammatory T cells in target organs

To provide additional confirmation that the protective effects observed with p28 antibody blockade were valid, we used IL-27⁺⁺ (WSX1⁺⁺)
Results

Figure 3. CD4+ T cells from IL-27−/− mice have a biased inflammatory phenotype. (A) Representative dot plots depicting CD44 and CD69 expression on CD4+ and CD8+ T cells from B6 and IL-27−/− mice. (B) Percentages of CD4+ and CD8+ T cells from wild-type (WT) or IL-27−/− animals (n = 3 per group) with naive, central memory (CM), or effector memory (EM) phenotypes. (C) Representative dot plot histograms showing CD3+, CD4+, CD69, CD25, CD127, CD103, GITR, and CTLA4 expression on gated CD4+ Foxp3− T cells from wild-type or IL-27−/− mice. (D) CD4+ and CD8+ T cell proliferation. (E) Percent CD4+ Foxp3− T cell proliferation. (F) Percent CD8+ T cell proliferation. (G) Survival. Data are from 2 experiments. *P < .05, **P < .01.
Results

Figure 4. Transplantation with IL-27R<sup>−/−</sup> marrow grafts reduces GVHD severity. (A) Representative dot plots depicting CD4<sup>+</sup> and CD8<sup>+</sup> expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells from B6 and IL-27R<sup>−/−</sup> mice. (B) Percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from wildtype or IL-27R<sup>−/−</sup> animals (n = 10 per group) with a naive, central memory (CM), or effector memory (EM) phenotype. (C) Representative dot plot showing CD25, CD44, CD69, CTLA4, CD103, GITR, and CD127 expression on gated CD4<sup>+</sup> Foxp3<sup>+</sup> T cells.
Results

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BLOOD, 20 OCTOBER 2016 • VOLUME 178, NUMBER 16

BLOKCLADE OF L-27P28 SIGNALING PREVENTS GVHD

animals in which there is specific deletion of the unique component of the heterodimeric IL-27R. Phenotypic characterization revealed that both CD4+ and CD8+ T cells from IL-27R−/− mice were also biased toward a central memory phenotype (Figure 4A) and that CD4+ Foxp3+ Tregs were phenotypically similar to wild-type Tregs (Figure 4C). Functional studies revealed no difference when compared with wild-type T cells with respect to alloantigen-induced proliferation or CD69 IFN-γ production (Figure 4D-E). There was, however, a modest reduction in the percentage of CD4+ GM-CSF+ and CD8+ TNF-α+ T cells after polyclonal stimulation (supplemental Figure 5A). More significantly, both CD4+ and CD8+ T cells from IL-27R−/− mice also produced less IFN-γ, and CD4+ T cells had reduced T-bet expression after TCR polarization (Figure 4F, supplemental Figure 6B), consistent with the described role of IL-27 in promoting TCR response.7 We observed that transplantation with marrow grafts from IL-27R−/− mice resulted in significantly prolonged survival compared with wild-type control animals (Figure 4G). There was also a significant increase in the absolute number of total CD4+ and CD8+ T cells, as well as CD4+ and CD8+ IFN-γ+ T cells, in the liver, lung, and colon (Figure 4H). Animals transplanted with IL-27R−/− BM and wild-type splenic cells had significantly worse survival compared with mice reconstituted with IL-27R−/− BM and IL-27R−/− splenic cells (supplemental Figure 7A). Conversely, IL-27R expression on BM alone had no adverse effect on overall survival (supplemental Figure 7B), indicating that IL-27 expression on T cells, and not BM cells, was critical for driving GVHD. Collectively, these results demonstrated that transplantation with IL-27R−/−, in contrast to IL-27R+/−, marrow grafts resulted in protection from lethal GVHD which supported the findings observed after p28 antibody blockade.

Blockade of IL-27P28 signaling augments the reconstitution of Tregs

We had demonstrated that antibody blockade of the IL-28R, which shares the gp130 subunit with IL-27, prevented GVHD by augmenting Treg reconstitution.11 Therefore, we conducted studies to determine whether protection by IL-27P28 antibody blockade similarly affected Treg recovery. Using Foxp3−/− reporter animals, we observed a significant increase in the absolute number of CD4+ and CD8+ Foxp3+ T cells in all tissues sites of mice treated with p28 antibody with the exception of CD8+ T cells in the liver (Figure 5A). Although the CD4+ Treg compartment is comprised of both natural Tregs (nTregs) and induced Tregs (iTregs), iTregs constitute the majority of this compartment. We therefore examined the effect of IL-27 on the induction of Tregs from the conventional T-cell pool. In vitro assays demonstrated that IL-27 had a dose-dependent reduction in the conversion of CD4+ and CD8+ Foxp3+ T cells from polyclonally stimulated CD4+ and CD8+ Foxp3+ T cells, respectively, indicating that IL-27 suppressed iTreg generation (Figure 5B). To determine whether this was operative in vivo, alloageneric recipients were transplanted with Rag-1− BM alone or together with sorted CD4+ Foxp3+ T cells and then treated with an isotype control or p28 antibody. Mice administered p28 antibody had a significantly increased number of both CD4+ and CD8+ Foxp3+ iTregs in all tissues sites, indicating that inhibition of IL-27 enhanced Treg reconstitution (Figure 5C). We then determined whether cohockleblock of IL-6 and IL-27 by antibody administration had any additive effects. These studies confirmed that administration of p38 antibody enhanced Treg reconstitution, whereas anti-IL-6R antibody treatment resulted in no increase in the absolute number of CD4+ or CD8+ Tregs. Thus, blockade of IL-27 was more potent than inhibition of IL-6 for enhancing Treg reconstitution when temporally concordant schedules were used. Notably, coadministration of both antibodies had no additive effect on CD8+ Treg numbers, but did result in a significant increase in CD4+ Tregs in the liver and lung compared with p38 antibody alone (Figure 5D). Finally, we conducted studies to determine whether the salutary effects of anti-IL-27P28 antibody blockade were attributable solely to the increase in Treg reconstitution because it was formally possible that IL-27 had effects on non-Treg populations. To address this question, recipients were transplanted with IL-27R−/− BM and CD8+ T cells from Foxp3+ cell mice which have no functional Tregs.7 We observed no difference in survival, indicating that the protective effect conferred by p28 antibody blockade was abrogated in the absence of functional Tregs (Figure 5E). Thus, these data provide evidence that GVHD protection from p28 antibody administration was attributable to augmented Treg reconstitution.

Lack of IL-27R expression of Treg augments their reconstitution and stabilizes Foxp3 expression posttransplantation

To further assess the effects of IL-27 signaling on Treg reconstitution, we created IL-27R−/− and Foxp3−/− mice to create a reporter mouse so that IL-27R−/− Tregs could be identified in vivo. We observed a significant increase in the absolute number of CD4+ and CD8+ Foxp3−/− T cells in mice reconstituted with IL-27R−/− Foxp3−/− grafts in all GVHD tissues when compared with animals receiving wild-type Foxp3+/+ grafts (Figure 6A), confirming that interruption of IL-27 signaling enhanced Treg reconstitution. To determine the functional competence of these cells, we conducted in vitro assays which demonstrated that CD4+ IL-27R−/−Foxp3−/− T cells were equally suppressive, or even superior at some dilutions, to wild-type CD4+ Foxp3+ T cells (Figure 6B). To further examine how IL-27R expression affected Treg biology, transplants were conducted in which animals received congenitally marked wild-type or IL-27R−/−CD4+ Tregs. We observed that the percentage and absolute number of adoptively transferred Tregs from IL-27R−/−Foxp3−/− mice that retained Foxp3 expression was significantly higher in all tissue sites when compared with Tregs from wild-type animals (Figure 6C-D). Furthermore, adoptive transfer of CD4+ IL-27R−/− Tregs resulted in significantly less GVHD-associated weight loss than wild-type Tregs, although there was no difference in survival (supplemental Figure 8). Finally, transplants were performed in which mice received adoptively transferred CD4+ Tregs from wild-type Foxp3+/+ animals and were then administered an isotype control or p28 antibody. Animals

Figure 4 (continued) from allogeneic or IL-27R−/− mice. (C) Column-purified T cells [5 × 106] from wild-type or IL-27R−/− mice were labeled with CFSE and cultured with allogeneic Rat-1 cells. CFSE+ cells (C.510−106) from wild-type or IL-27R−/− mice were labeled with CFSE and cultured with allogeneic Balanced Confluent density cells (DCs) (1 × 105) for 4 days. The percentage of CFSE− CD4+ and CD8+ T cells in the absence of presence of DCs is shown for replicate experiments (n = 3). Data are presented as mean ± SEM. (D) Column-purified T cells [5 × 106] from allogeneic or IL-27R−/− mice were cultured with PMA and ionomycin for 1 hr. Representative dot plots depicting the percentage of CD4+ or CD8+ IFN-γ+ T cells that coexpressed Foxp3 and T-bet and the percentage of CD4+ or CD8+ T-bet+ T cells that coexpressed IFN-γ and Foxp3 are shown (n = 5–6 per group). (E) Tissue recipients were transferred with 106 BM alone (n = 10), IL-27−/− BM (n = 10), IL-27−/− BM and IL-27−/− spleen cells (n = 10), or IL-27−/− BM and IL-27−/− spleen cells plus (n = 10) (adjusted to yield an up T-cell dose of 1.5 × 106 cells). Overall survival is depicted. Data are from 3 experiments. (F) Lethally irradiated Wild-type recipients were transferred with 106 BM alone (Black bars, n = 5), IL-27−/− BM (Gray bars, n = 5), IL-27−/− BM and IL-27−/− spleen cells (n = 5), or IL-27−/− BM and spleen cells (Gray bars, n = 5). The absolute number of lymphocytes and spleen cells are depicted. Data are from 3 experiments. *P < 0.01; **P < 0.001; ***P < 0.0001.
Results

Figure 5: Blockade of IL-27 signaling augments reconstitution of Foxp3-expressing Tregs. (A) Balb/c mice were transplanted with B6 Foxp3<sup>EGFP<sup>T</sup></sub> BM and spleen cells and then treated with either an isotype control (a) or 28 IgG antibody. Animals were killed 14 days posttransplantation and the absolute number of CD4<sup>+</sup> and CD8<sup>+</sup> Foxp3<sup>+</sup> T cells in the spleen, liver, lung, and colon are shown. (B) Sorted CD4<sup>+</sup> or CD8<sup>+</sup> Foxp3<sup>EGFP<sup>T</sup></sub> T cells were cultured with anti-CD4/CD8 antibodies, IL-2, and TGF-β alone, or together with graded doses of IL-27. The percentage of CD4<sup>+</sup> and CD8<sup>+</sup> Foxp3<sup>+</sup> T cells is depicted. (C) Balb/c mice were transplanted with B6 Rep-1 BM alone or together with sorted CD4<sup>+</sup> (6.4 × 10<sup>6</sup>) and CD8<sup>+</sup> (3.6 × 10<sup>6</sup>) Foxp3<sup>EGFP<sup>T</sup></sub> T cells and then treated with either an isotype control (a) or pIL-12 (b) antibody. Animals were killed 14 days posttransplantation and the absolute number of CD4<sup>+</sup> and CD8<sup>+</sup> Foxp3<sup>+</sup> T cells in spleen, liver, lung, and colon is shown. (D) Balb/c mice were transplanted with B6 Foxp3<sup>EGFP<sup>T</sup></sub> BM and spleen cells and then treated with either an isotype control (a, n = 10), anti-IL-6 (a, n = 10), p18 (a, n = 10), or combination of p18 and anti-IL-4 (a, n = 10) antibodies. The absolute number of CD4<sup>+</sup> and CD8<sup>+</sup> Foxp3<sup>+</sup> T cells in spleen, liver, lung, and colon 2 weeks posttransplantation is depicted. (E) Balb/c mice transplanted with B6 Rep-1 BM alone (a, n = 5) or with 6.4 × 10<sup>6</sup> CD4<sup>+</sup> EGFP<sup>T</sup> and 3.6 × 10<sup>6</sup> CD8<sup>+</sup> EGFP<sup>T</sup> T cells from Foxp3<sup>EGFP<sup>T</sup></sub> animals that had been reconstituted with 4.0 × 10<sup>6</sup> to 6.0 × 10<sup>6</sup> spleen cells from B6.53.3, Foxp3<sup>EGFP<sup>T</sub></sup> (CD45.3) animals 1 to 2 days after birth to prevent the development of lethal autoreactivity. Mice transplanted with these T cells were treated with either isotype control (a, n = 10) or p18 antibody (b, n = 10) on days 0 and 6 posttransplantation. Overall survival is depicted. Data are from 2 experiments for each panel. *P < .05, **P < .01, ***P < .001. EGFP, enhanced green fluorescent protein; TGF, transforming growth factor.
Results

Figure 6.
administered p28 antibody had a significantly increased absolute number of CD4+ Tregs (Figure 6D) and there was a higher percentage of Tregs that maintained Foxp3 expression compared with isotype antibody-treated mice (Figure 6D), confirming data observed with congenerically marked IL-27Rα−/−Foxp3ΔCD4+ T cells.

**IL-27 signaling blockade has differential effects on IL-10 production by conventional T cells and Tregs**

Because IL-10 is one mechanism by which CD4+ Tregs suppress GVHD15 and IL-27 has been shown to enhance T-cell-derived IL-10 secretion in nontransplant models,23,24 we examined whether IL-27 blockade adversely affected IL-10 production by Tregs. To confirm that IL-27 augmented IL-10 production, T cells from IL-10R−/−Foxp3GFP dual reporter mice were activated with anti-CD3/CD28 antibodies and IL-2 alone or in the presence of graded doses of IL-27. We observed a dose-dependent increase in the percentage of conventional CD4+ and CD8+ IL-10+ T cells with the effects most pronounced for CD8 T cells (Figure 7A). IL-27 also had a statistically significant effect on Treg production of IL-10, but this was biologically negligible (Figure 7B). To confirm that IL-10 detected in these reporter assays was biologically active, we performed enzyme-linked immunosorbent assays which demonstrated elevated levels of IL-10 in ex vivo samples of polyclonally stimulated CD4+ and CD8+ T cells (Figure 7C). To determine whether blockade of IL-27 reduced IL-10 production in vivo, Balb/c recipients were transplanted with marrow grafts from IL-27R−/−Foxp3GFP reporter animals. Mice administered p28 antibody had a significant reduction in the frequency of conventional CD4+ and CD8+ IL-10+ T cells in all tissue sites (Figure 7E). Furthermore, the absolute number of conventional CD4+ IL-10+ T cells was reduced in the liver and lung, whereas the number of CD8+ IL-10+ T cells was decreased in the liver (Figure 7E). In contrast, there was an increase in the frequency of CD4+ and CD8+ Foxp3+ IL-10+ T cells in nearly all tissue sites, as well as an increased absolute number of CD4+ and/or CD8+ IL-10+ Tregs in the colon and liver (Figure 7F). These results indicate that blockade of IL-27 signaling had differential effects on IL-10 production in conventional T cells vs Tregs, and that IL-10 production by Tregs was not adversely affected by inhibition of IL-27.

**Discussion**

IL-27 is a member of the IL-12 cytokine family which plays a pivotal role in the biology of GVHD. Two of the most well-studied cytokines in this family, IL-12 and IL-23, have been demonstrated to mediate proinflammatory effects, whereas IL-15 has been reported to have an anti-inflammatory role in GVHD15,28 as well as other transplant models.62 The goal of these studies therefore was to define whether IL-27 had a proinflammatory or anti-inflammatory role in GVHD biology. Unexpectedly, as we conducted these studies, we observed discrepant results in animals that were reconstituted with naive grafts from IL-27p28−/− animals vs mice that received IL-27p28−/− grafts or were administered a p28-specific antibody with respect to protection from lethal GVHD. Whereas transplantation with IL-27p28−/− grafts exacerbated GVHD, mice reconstituted with IL-27Rα−/− grafts or those treated with p28 antibody had significantly reduced GVHD. A potential explanation for these results was that T cells from IL-27Rα−/− mice arming in this transgenic environment were more biased toward a proinflammatory phenotype when compared with wildtype T cells. Prior studies by Zhang and colleagues had shown that CD4+ T-cell production of IFN-γ was significantly augmented in mice in which there was conditional deletion of IL-27 from dendritic cells.44 They proposed that the lack of endogenous IL-27 altered T-cell development within the thymus resulting in hyperactive T cells. Toward that end, we observed that there was a higher percentage of CD8+ T cells, but not CD4+ T cells, that secreted IFN-γ post polyclonal stimulation or TCR polarization in vitro, and an increase in the absolute number of CD8+ IFN-γ+ T cells in GVHD tissue sites. Moreover, donor CD8+ T cells from IL-27Rα−/− animals induced greater GVHD lethality than wildtype CD8+ T cells when cotransferred with wild-type CD4+ T cells, providing in vivo evidence that the absence of APC-derived IL-27 led to dysregulation of the CD8+ T-cell compartment. Notably, this was not observed in T cells from IL-27Rα−/− mice where APC production of IL-27 was intact and which induced significantly less GVHD, supporting the p28 antibody results. In fact, both CD4+ and CD8+ IL-27Rα−/− T cells produced less IFN-γ after TCR polarization which is consistent with prior studies.12,26,27,36,37,45,46 Thus, even though CD8+ T cells from both IL-27Rα−/− and IL-27p28−/− mice would be similarly primed in vivo, CD8+ IL-27Rα−/− T cells had a more exaggerated IFN-γ response after exposure to the GVHD inflammatory milieu which included IL-27 produced by donor BM-derived APCs. Collectively, we believe that the more clinically relevant studies using p28 antibody administration, coupled with the confirmatory results obtained using IL-27Rα−/− mice, validates that IL-27 plays a proinflammatory role in GVHD biology.

Protection from lethal GVHD by interruption of IL-27 signaling through either antibody-based or genetic approaches was associated with a significant increase in Treg reconstitution in all GVHD target organs. Notably, all Treg populations were increased, including CD4+ Tregs which have also been shown to play a role in GVHD prevention.63 Because IL-27 has direct effects on conventional T cells, it was formally possible that the protective effects were attributable primarily to a reduction in TCR cells and were not the consequence of augmented Treg reconstitution. To determine whether Treg reconstitution was directly responsible for the reduction in GVHD, we conducted studies in which animals received marrow grafts devoid of all Tregs. Under these conditions, administration of p28 antibody had no protective effects, providing proof that the

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**Figure 6. Absence of IL-27R expression on Tregs enhances reconstitution and stabilizes Foxp3 expression.** (A) Spleen cells from IL-27R−/−Foxp3GFP mice were transplanted with 36.6×103 BM cells (black bars, n = 5) or 1.7×10^7 Foxp3GFP BM (light gray bars, n = 5). (B) Serum levels of IFN-γ in the spleen (medium gray bars, n = 9) or 1.7×10^7 Foxp3GFP BM and spleen cells (gray bars, n = 9) (adjusted to yield an 80% T-cell dose of 0.7×10^6 cells). The absolute number of Tregs in the spleen, lung, and colon is depicted. Data are results from 2 experiments. (C) BM from TCRβ-labeled 96 Thy-2+ T cells (n = 10) were co-injected with BDF1 CD4+ T cells (8×10^6) alone or in the presence of varying ratios of sorted BM Foxp3GFP+ or CD4+ IL-27Rα−/−Foxp3GFP+ Treg and 5 days in triplicate wells. Control wells were depleted of black bars. Data are presented as the mean percentage ± SD of TCM Cell Trace Violet expressing cells from an representative of 3 experiments with similar results. (D,E) BM- and spleen cells were transplanted with 86.4% BM and spleen cells (8×10^6) × 10^6 T cells with either 0.6 × 10^3 CD3+CD4+ T cells or CD4+ Thy-2+Foxp3GFP+ Tregs. Representative data from the percentage of Thy-2+Foxp3GFP+ Tregs in the spleen, lung, and colon that were retained expression of Foxp3 2 weeks post-transplantation in shown in panel C. The percentage and absolute number of CD4+ Thy-2+Foxp3GFP+ T cells in the same tissue sites are depicted in panels D and E, respectively. (F,G) Spleen cells from IL-27Rα−/− mice were transplanted with 86.4% BM and spleen cells (6×10^6) × 10^6 T cells along with 0.6 × 10^3 sorted Thy-2+CD4+ Foxp3GFP+ Tregs. Cohorts of mice that received either an isotype control or p28 antibody on days 5 and 6. The absolute number and percentage of Thy-2+CD4+ Foxp3GFP+ T cells in the spleen, lung, and colon are depicted in panels F and G. Data are from 2 experiments. *P < .05, **P < .01, ***P < .001.
Results

Figure 7.
Results

Prior studies have demonstrated that CD4⁺ Tregs express the IL-27R, although the functional significance of this has been controversial. Cox and colleagues reported that IL-27 limited Treg conversion in a T-cell transfer model of colitis as well as in an ovalbumin-dependent tolerization model. These findings are consistent with previous studies that had shown that IL-27 inhibited the conversion of CD4⁺ Foxp3⁻ into CD4⁺ Foxp3⁺ T cells both in vitro and in vivo. Conversely, Kim and coworkers noted that transfer of CD4⁺ CD25⁺ T cells from IL-27R⁻⁻ mice induced worse colitis when compared with recipients of wild-type CD4⁺ CD25⁺ T cells. This was associated with reduced Treg numbers in animals reconstituted with IL-27R⁻⁻ T cells and the authors concluded

Figure 7. Antibody blockade of IL-27 has differential effects on IL-10 production in CD4⁺ and CD8⁺ conventional T cells vs Tregs. (A-B) Anti-CD25/28 antibody activated CD4⁺ or CD8⁺ conventional T cells (I locus) or Tregs (B) from B6IL-10-kd23W68 (-/−) reporter mice cultured in L-2, 20 U/mL IL-2. (C-D) Groups of B6IL-10-kd23W68 (-/−) reporter mice received 10⁶/animal naive (CN) or CD4⁺ conventional T cells from L-2, 20 U/mL IL-2 cultured in the presence of graded doses of IL-27 (10⁶-10⁴ U/mL) for 3 days. The percentage of IL-27 transfer conventional T cells is depicted. (E) CD4⁺ and CD8⁺ T-cell populations in C57BL/6J mice treated with anti-CD25/28 antibody on days 0 and 6. Representative dot plot showing the percentage of CD4⁺ or CD8⁺ T cells expressing Foxp3 and IL-10 in the spleen, liver, lung, and colon 10 days post-transplantation (D). The frequency and absolute number of conventional T cells expressing IL-10 in the spleen, liver, lung, and colon on day 10 are depicted. *P < .05, **P < .01, ***P < .001.
Results

that this was due to a requirement for IL-27 to maintain Treg survival. In the current study, we crossed IL-27"""" mice with Foxp3"""" animals to create a reporter strain that allowed for the specific detection and selection of CD4"""" IL-27"""" Foxp3"""" Tregs. These mice revealed that transplantation with marrow grafts from IL-27"""" Foxp3"""" mice resulted in a significant increase in the absolute number of CD4"""" and CD8"""" Tregs in GVHD target tissues. Moreover, the adoptive transfer of Tregs that lacked expression of the IL-27R, or transfer of wild-type Tregs followed by administration of p8 antibody, resulted in a significant increase in the absolute number of these cells in the same tissue sites.

Perhaps the most notable finding was that inhibition of IL-27 signaling resulted in greater stability of Foxp3 expression in CD4"""" Tregs. Preclinical studies have shown that the inflammatory milieu arising in the setting of GVHD can lead to loss of Foxp3 expression in both nTreg and Treg populations. These so-called eTregTs can thus acquire the capability to produce Th1-type cytokines, such as IFN-γ, and subsequently contribute to pathological damage. This raises a legitimate concern that strategies to expand Treg ex vivo for subsequent adoptive transfer may result in unwanted inflammatory effects as a consequence of Foxp3 instability. Our observation that antibody blockade of IL-27 not only augmented Treg reconstitution, but also stabilized Foxp3 expression, suggests that this might be a viable clinical approach to promote in vivo Treg reconstitution without the requirement for more costly ex vivo expansion strategies, as well as minimize the potential of Treg conversion to a more pathogenic cellular phenotype.

IL-10 has been shown to be one of the primary mechanisms by which Tregs mitigate the severity of GVHD, and absence of de novo-derived IL-10 is associated with a significant increase in GVHD-associated mortality. Thus, IL-10 plays a critical role in the regulation of GVHD. IL-27 has been shown to induce secretion of IL-10 by T cells, thereby raising the possibility that blockade of IL-27 might deleteriously affect regulation of GVHD by inhibiting this cytokine, even though there was an increase in overall Treg numbers. In fact, we did observe that blockade of IL-27 reduced the frequency of conventional CD4"""" and CD8"""" IL-10"""" T cells in all tissue sites with absolute reductions observed in the liver and lung for CD4"""" """" T cells, and the liver alone for CD8"""" """" T cells. However, antibody-mediated inhibition of IL-27 resulted in an increase in the frequency of CD4"""" and CD8"""" Foxp3"""" IL-10"""" T cells in nearly all tissue sites, as well as an increased absolute number of CD4"""" and CD8"""" IL-10"""" T cells in the colon and liver. Thus, blockade of IL-27 had differential effects on conventional T cell vs. Treg populations, and preserved a critical mechanisms pathway by which Tregs have been shown to suppress GVHD. These results are consistent with our in vitro studies which demonstrated that IL-27 resulted in a significant increase in the percentage of conventional CD4"""" and CD8"""" T cells that produced IL-10, but had only very modest effects on Tregs, leading one to surmise that blockade of IL-27 would have minimal effects on Treg-induced IL-10 production in vivo which is what we observed. The fact that a reduction in the absolute number of IL-10-producing conventional T cells had no adverse effect on GVHD is consistent with prior studies in which effecter T-cell production of IL-10 was shown not to have a regulatory role in mitigating GVHD.

In summary, we have identified that blockade of the IL-27 signaling pathway resulted in protection from lethal GVHD and that was attributable to enhanced reconstitution of all Treg subsets as well as stabilization of Foxp3 expression. Additionally, inhibition of IL-27 selectively reduced IL-10 production in conventional T cells, but not Tregs, thereby preserving the ability of Tregs to suppress GVHD through this mechanistic pathway. The targeting of IL-27 therefore represents a novel clinical strategy for the in vivo expansion of Tregs and subsequent prevention of GVHD, and provides additional support for the critical role that cytokines in the IL-6 and IL-12 families play in GVHD biology.

Acknowledgments

This work was supported by grants from the National Institutes of Health, National Heart, Lung, and Blood Institute (HL046603 and HL126166) and by awards from the Midwest Athletes Against Childhood Cancer Fund (W.R.D.). L.B. was supported by a grant from Wallonia Brussels International.

Authorship

Contribution: L.B. performed animal studies and flow cytometric analysis, and helped write the manuscript; K.A. performed research, analyzed data, generated figures, and helped write the manuscript; V.Z., C.Y.-Y., M.G., and R.M. performed research and analyzed data; R.K. performed pathological analysis of all tissue samples; D.E. and B.L. conducted the histological analysis; I.S., D.C., N.G., C.B.W., and J.v.S. provided critical reagents and edited the paper; and W.R.D. developed the overall concept, designed experiments, supervised the study, and wrote the manuscript.

Conflict-of-interest disclosure: N.G. is a present employee of Genentech, a member of the Roche group. D.C. is employed by Merck Research Labs. The remaining authors declare no competing financial interests.

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TLR7 ligand R848 prevents mouse GVHD and cooperates with anti-IL-27 mAb for maximal protection and Treg upregulation

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Funding information

This work was supported by the Belgian National Fund for Scientific Research (FNRS), the "Fondation Contre le Cancer" (FCC)(2010-165), the Joseph Maisin Fund and the Interuniversity Attraction Pole of the Belgian Federal Science Policy.

Key points

TLR7 ligand R848 impairs mouse acute GVHD without preventing donor hematopoietic cell implantation. It inhibits IFNγ, IL-27 and TNFα production while upregulating active TGF-β1 and Foxp3+ Tregs. It inhibits, allo-antigen presentation by dendritic cells and T cell allo-responsiveness in a process dependent on type I IFNs. Further inhibition of IL-27 by p28-specific mAb cooperates with R848 for optimal protection and Treg expansion.
Results

Abstract

In spite of considerable therapeutic progress, acute graft versus host disease (GVHD) still limits allogeneic hematopoietic stem cell transplantation. We recently reported that mouse infection with nidovirus LDV impairs disease in non-conditioned (nc) B6→B6D2F1 GVHD. As this virus activates TLR7, we tested TLR7 ligand R848 in this model and observed complete survival if donor and recipients were treated before transplantation. Mixed lymphocyte culture (MLC) performed 48 h after R848-treatment of normal mice demonstrated that both T cell alloresponsiveness and antigen presentation by CD11b+ and CD8α+ dendritic cells were inhibited. These inhibitions were dependent on IFNAR-1 signaling. In B6→ncB6D2F1 GVHD, R848 decelerated, but did not abrogate, donor T cell implantation and activation. However, it decreased IFNγ, TNFα and IL-27 while upregulating active TGF-β1 plasma levels. In addition, donor and recipient Foxp3+ Treg cell numbers were increased in recipient mice and their elimination compromised disease prevention. R848 also strongly improved survival of lethally irradiated BALB/c recipients of B6 HC graft (B6 →8GyBALB/c) and this also correlated with an upregulation of CD4 and CD8 Foxp3+ Tregs that could be further increased by IL-27 inhibition. The anti-IL-27-R848 combination showed strong synergy in preventing disease in the B6 →B6D2F1 model when recipients were sublethally irradiated. We conclude that R848 modulates multiple aspects of GVHD and offers potential for safe allogeneic bone marrow transplantation that can be further optimized by IL-27 inhibition.
Introduction

Allogeneic hematopoietic cell transplantation (aHCT) is an important therapeutic option for a wide range of malignant and non-malignant disorders, including acquired and genetic anomalies (1-5). However, it remains plagued by bone marrow failure or graft versus host disease (GVHD), which develop in approximately 50% of aHCT recipients (6, 7). With a death rate in the range of 15% (8), acute GVHD thus remains a major medical challenge, especially in older patients and in steroid-resistant cases where the mortality reaches 90% (9). An extended review on GVHD biology and therapy, published in 2012, listed 39 different therapies for the acute form of the disease but concluded “systemic corticosteroid therapy, despite its major shortcomings, remains the first standard therapy for GVHD. New and improved therapies are therefore desperately needed”(10).

A hallmark of aGVHD is the release of inflammatory cytokines and their pathogenic contribution has been demonstrated for TNFα (11), IL-6 (12), IL-23 (13) and more recently IL-27 (14) (15). Analysis of the consequences of IL-27 inhibition demonstrated the essential role of both natural and induced Tregs in controlling GVHD pathology and their inhibition by IL-27. These results are in line with the protective activity of Tregs reported previously (16-18) and offer a novel option for their in vivo upregulation for GVHD prevention.

We recently made the surprising observation that lactate dehydrogenase elevating virus (LDV), a single stranded positive-sense RNA enveloped mouse nidovirus, enhanced survival in the lethal form of acute GVHD induced by B6 spleen cell injection into non-conditioned B6D2F1 recipients (19). In this model, which is mainly characterized by severe bone marrow failure (20) and liver destruction, LDV protection correlated with a
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transient impairment of antigen presentation by DCs and with alterations in T cell allo-responsiveness, both dependent on IFNAR-1 signaling, in agreement with the reported GVHD inhibition by Type I IFNs (21).

As LDV activates TLR7 (22), we considered the possibility that TLR7 agonist resiquimod (R848) (23), an imidazoquinoline originally developed as an antiviral agent (24) and an immune adjuvant (25), could replicate the protective effects of the virus. This idea is somewhat counterintuitive since R848 has adjuvant activity, promotes Th1 immune reactions (26) through activation of myeloid differentiation primary gene 88 (Myd88) (23), stimulates iNKT cells (27) and anti-tumor immunity (28) while inhibiting allergen induced Th2 responses (29). The activity of R848 is, however, not limited to stimulating Th1 cells. It also stimulates B cell proliferation and antibody production (30) as well as regulatory T cell-mediated TGF-β-dependent suppression of allergic asthma (31). Moreover, it induces a transient immune incompetence resulting from leukocyte trapping on epithelial cells in peripheral organs due to upregulation of ICAM-2 and P-selectin (32) and daily administration of R848 to otherwise non-manipulated mice for one week induces an HIV-associated pathology with lymphopenia, elevated inflammatory cytokines and splenomegaly (33).

Based on these complex properties, it was hard to predict the effect of R848 on GVHD but given the positive consequences of a transient acute TLR7 activation by LDV, we explored the effects of R848 administration in several aHCT models. Depending on the model, we obtained complete or partial protection that could be further improved by IL-27 inhibition.
Results

Materials and Methods

Mice

Mice were bred under SPF conditions at the animal facility of the Ludwig Cancer Research Brussels Branch under the direction of Guy Warnier (DVM). Experimental protocols and animal handling were approved by the ethical committee of the Medical Faculty of the Université de Louvain (accreditation no: 2016/UCL/MD/010). IFN-α/βR-/- 129/Sv mice (IFNAR-1/-) were a gift of Dr. M. Aguet (34).

GVHD experiments

Parent to F1 GVHD models: on day -1, B6xDBA/2 F1 (B6D2F1) recipient mice were left untreated or irradiated with 5 Gy from a ^{137}Cs source before aHCT. On day 0, they received an i.p injection of 50-70 x10^6 C57Bl/6 (B6) spleen cells and, for the irradiated mice, 10 x10^6 of B6 CD3ε+ T cell-depleted BM cells (TCD-BM). Donor and recipient mice were treated with two i.p. injections of Resiquimod R848 (Cat.ALX-420-038-M005, EnzoLifeSciences, NY, USA), 25ug/mouse, 48 and 24 hours before B6 cell implantation. Irradiated B6D2F1 mice received i.p injection of mouse αIL-27p28 antibody (Ab) (0.5 mg MM27.7B1) (35) at day 0 and 6. Lethally irradiated model: BALB/c recipient mice were irradiated with 2x4Gy at 6 hour interval and treated or not with R848 (25 µg/mouse) 48 hours before and immediately after transplantation of 2.5x10^6 CD5+ splenocytes + TCD-BM cells from B6 mice. In addition, recipients were treated or not with αIL-27p28 Ab (0.5 mg) at days 0 and 6. In some experiments, mice were also treated with two i.p. injections of anti-CD25 Ab (clone: PC61) (generated in-house from hybridomas obtained from ATCC, VA, USA (Loventhal 1985)) 400µg/mouse, 6 days before and 1 day after B6 cell transfer. All experiments were
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performed on adult mice with a weight of ± 20 g. Mice were monitored for survival and weight loss every other day. Mice were bled on the indicated days to monitor plasma cytokines and, at the experimental endpoint, euthanized for spleen and liver analyses.

Liver Histology

For histo-pathology analyses, paraffin tissue blocks of 4% paraformaldehyde fixed liver were prepared using routine methods and six micron consecutive sections were made. The sections were stained with hematoxylin and eosin (H&E) and scanned with Pannoramic P250 digital slide scanner (3D HISTECH).

Mixed lymphocyte culture

MLC was carried out by incubating 1.25x10^6 responder spleen cells/ml with an equal concentration of irradiated (30 Gy from a ^{137}Cs source) adherent spleen cells. In some cases, responders were CD4 T cells purified by MACS from spleens and seeded at a density of 1x10^6 cells/ml. Adherent cells were obtained by coating 1x10^6 splenocytes in a 96-well flat bottomed microtiter plate for 1.5h and removing non-adherent cells by washing the microplate twice with PBS (37°C). DC subpopulations were cultured at 10^4 cells per well. Proliferation was measured after two days by incubation with [^{3}H]-Thymidine at 1 µCi (0.037 MBq)/well for a further 18h. [^{3}H]-Thymidine incorporation was measured using a scintillation counter (Packard Microplate Scintillation Counter).

Flow cytometry and cell sorting

To determine chimerism, splenocytes were stained with anti-H-2D^d-FITC (clone: 34–2–12) and anti-H-2D^b-PE (clone: KH95) (all from Biolegend, San
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Diego, CA). Spleen cells were also labelled with anti-CD4 (clone: RM4-5 and GK1.5), anti-CD8α (clone: 53-67), anti-TCR-β (clone: H57-597), anti-CD44 (clone: IM7), anti-CD62L (clone: MEL-14), anti-CD69 (clone: H1.2F3), anti-CD25 (clone: PC61) and anti-LAP (clone: TW7-16B4) Abs, all from Biolegend, and a viability marker (LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit, Life Technologies, Eugene, OR). Treg cells were determined in spleens according to manufacturer’s instructions using a kit from eBioscience (San Diego, CA). For MACS cell purification or depletion, we used anti-CD5 (cat. 130-049-301) and anti-CD3ε (cat. 130-094-973) microbeads (Miltenyi Biotec Bergisch Glabach, Germany). For in vitro functional assays, DC subpopulations were purified from spleens as previously described (19). All cells were acquired using a FACS-LSRFortessa according to BD bioscience protocols and analysed by FlowJo software version 9.8.1.

Cytokine measurements

Cytokine production was measured in cell culture supernatants and plasma. ELISA specific for murine IFNγ (R&D Systems, MN, USA) and TNF-α (LifeTechnologies, MD, USA) were performed according to manufacturer’s instructions. IL-27p28, active TGF-β1 and TGF-β3 were measured using mAbs generated in our laboratory as previously described (14). In all ELISAs, biotinylated detection Abs were used followed by avidin-HRP (Biolegend). Absorbance readings were made at 450 nm, using a 96-well plate spectrophotometer (VERSAmax, Molecular Devide). Detection limits are less than 10 pg/ml.

RT-qPCR

Total RNA was isolated from spleen and liver with TriPure isolation reagent (Roche, IN, USA). One µg of total RNA was reverse transcribed using
oligo(dT) primer (Eurogentec, Vilnius, LT) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative PCR (qPCR) amplifications were performed on cDNA obtained from 25 ng of total RNA, using primer sets and TaqMan probes (Eurogentec) or MasterMix for SYBR Green (Eurogentec). Primers and probes used for $\beta$-actin were 5'-CTCTGGCTCCTAGCACCATGAAG-3', 5'-GCTGGAAGGTGGACAGTGAG-3'and 5'-TCGCTGCTCCATCCTGCCG-3'. Primers and probe used for Ifn$\gamma$ were 5'-TCAAGTGGCATAGATGGAGAAAG-3', 5'-TGGCTCTGAGGATTTGTTTGCATG-3' and 5'-TCACCATTGGTTGCTCCTCCAG-3'. Primers for SAA1/2 were 5'-TCTGGCTCCTGCTGAGGGA-3' and 5'-TGGCTTCCTGTCAGCCATG-3'. Primers for SAA3 were 5'-GCTGGTCAAGGGTCTAGAGAC-3' and 5'-GATGACTTTAGCGCCAGGC-3'. Samples were first heated for 10 min at 95°C before amplification as follows: 40 cycles of two-step PCR program at 95°C for 10 s and 60°C for 1 min. For SYBR Green qPCR, melting point analysis was carried out by heating the amplicon from 60 to 95°C. A standard curve with known concentrations of a cloned cDNA fragment was used for each gene.

**CFSE staining**

CD5+ T cells were suspended at 20x10^6 cells/ml in serum-free media and CFSE (Invitrogen, Oregon, USA) was added at 0.5uM final concentration. Cells were incubated at 37°C for 10 minutes and reaction was stopped with the same volume of cold FCS and washed in media containing 3% FCS. Cells were injected i.p. in recipient mice and after 6 days, they were analysed on a FACS-LSRFortessa.
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Statistical analysis

Statistical analysis was performed with Prism 5 (Graphpad Software, La Jolla, CA) using non-parametric tests (Kruskal–Wallis or Mann–Whitney), parametric test (Bonferroni’s multicomparison), and Log-rank Test for survival curves.

Results

R848 prevents lethal parent to F1 non-conditioned GVHD (ncGVHD)

The effect of R848 was first tested in ncGVHD to avoid the inflammatory cytokine burst induced by host irradiation. The agent was administered to recipient B6D2F1 and/or donor B6 mice 24 and 48 h before donor spleen cell injection. Data pooled from 5 experiments showed 100 % mortality in control mice by day 25. R848 treatment of either recipient or donor resulted in survivals of 40 and 60 %, respectively while combined treatment of both resulted in 100 % long-term survival (Fig.1A). Morbidity evaluated by weight loss was also totally suppressed after donor and recipient treatments (Fig.1B). Timing of R848 administration was important since too early (6-5 days before transplantation) or too late (5-6 days after transplantation) treatments were not protective (Fig.1C). B6→ncB6D2F1 transplantation is also characterized by liver inflammation and destruction. R848 treatment minimized hepatocyte destruction but mononuclear cell infiltration was still present (Fig.1D). This tissue protection was confirmed by a complete suppression of serum amyloid A (SAA)1/2 and SAA3 mRNA expression in liver cells (Fig.1E).
Results

B6→ncB6D2F1 transplantation results in almost complete spleen cell destruction. When host B6D2F1 spleen cells were counted 14 days after B6 cell transplantation, their numbers had decreased from ± 50 to ± 2 x10^6 in the untreated group but increased to ± 80 x10^6 in R848-treated mice. Spleen cell implantation by R848-treated B6 donors was low after 14 days, approximately 4 x10^6 cells, but increased with time, resulting in a permanent chimerism reaching 20 x10^6 B6 cells per spleen after 100 days (Fig.1F).

**R848 treatment inhibits production of IFNγ, TNFα and IL-27 but stimulates active TGF-β1 secretion during B6→ncB6D2F1 GVHD**

B6→B6D2F1 ncGVHD is characterized by high concentration of plasmatic IFNγ and IL-27 and they reached maxima at day 10 and returned to nearly undetectable levels by day 14. R848 treatment essentially abolished these cytokine peaks (Fig.2A). RNA analysis confirmed upregulation of IFNγ expression in spleen and liver in untreated mice and its complete silencing by R848 (Fig. 2B). Another cytokine contributing to GVHD pathology is TNF α (36). A significant increase of this cytokine was detected in plasma of untreated ncGVHD mice at days 10 and 14 and this was also suppressed by R848 treatment (Fig. 2A). Given the potential implication of TGF-β in the control of GVHD (37, 38), we also measured active TGF-β1 and TGF-β3 by ELISAs selectively detecting the active forms of these cytokines and observed a strong upregulation of the former (Fig.2A), but not the latter (data not shown) after R848 treatment. As shown in Fig.2A, active TGF-β1 was upregulated from day 6 to day 14, but was no longer detectable at day 50 (data not shown). R848 treatment thus abrogates the proinflammatory cytokine response in favor of an increase of active TGF-β1.
Results

R848 treatment before HCT inhibits T cell allo-responsiveness and MHC presentation by cDCs through type I IFN signaling

As mentioned above, optimal prevention of B6→ncB6D2F1 GVHD requires treatment of donor and recipient with R848. Since host T cells are not reactive against the implanted parental cells, the action of R848 on the F1 partner is likely on antigen presentation. To assess the influence of R848 on this process and on T cell responsiveness, normal B6D2F1 and B6 mice were injected with R848 or PBS for two consecutive days before spleen cell collection. B6 spleen cells were then incubated with B6D2F1 irradiated adherent spleen cells as a source of APC. When performed with control mice, this combination induced strong proliferation and IFNγ production. R848 treatment of either B6D2F1 stimulating or B6 responder mice inhibited proliferation, indicating that a 48h treatment with R848 of otherwise non-manipulated mice impaired both antigen presenting and responder cells. The same inhibition was observed for IFNγ production (Fig.3A).

TLR7 activation leads to production of type I IFNs (23). We could not detect biologically active IFNs in the plasma in R848-treated ncGVHD mice (data not shown) but we tested IFN implication in R848-mediated T cell suppression in allogeneic MLC (A-MLC) using spleen cells from 129/Sv (H-2Db) or 129/Sv-IFNAR-1−/− mice as responders and B6D2F1 irradiated adherent spleen cells as APC. 129/Sv and 129/Sv-IFNAR-1−/− mice were treated twice for two consecutive days with R848 and spleen cell proliferation and IFNγ production were compared to control conditions. This experiment confirmed the inhibitory effect of R848 on the allogeneic responder cells in a different mouse strain and showed that, in 129/Sv-IFNAR-1−/− responder cells, proliferation and IFNγ production were not inhibited (Fig.3B). R848-induced inhibition was not correlated with an
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increase in FoxP3+ Tregs (Fig. 3C) and depletion of Treg with anti-CD25 PC61 Ab before R848 treatment did not prevent the inhibition of IFNγ production during A-MLC (Fig.3D).

We previously showed that TLR7 viral stimulation transiently inhibits conventional DCs (cDCs), the only splenic cells able to activate allogeneic T cell response in vitro (19). To evaluate the effect of R848 on allogeneic antigen presentation in A-MLC, CD11b+ cDCs, CD8α+ cDCs and pDCs were purified from 129/Sv mice and co-cultured with FVB (H-2q) CD4 T cells. CD11b+ and CD8α+ cDCs were the main stimulating cells in MLC and this capacity was impaired in mice treated with R848 (Fig. 3F). This suppression of APC was also IFNAR-1-dependent (Fig.3G).

These data indicate that at the time of donor cell transfer, R848 administration inhibited both antigen stimulation by cDC and T cell responsiveness in a type 1 IFN-dependent process. This impaired T cell allo-responsiveness induced by R848 after 48 hours, did not involve Tregs.

**R848 treatment impairs effector donor T cells and increases Tregs in B6→ncB6D2F1 GVHD**

To evaluate the effect of R848 on responder T cells during GVHD, we first used CFSEabeled CD5 B6 cells. Six days after transplantation, CFSE staining dropped considerably in B6 cells transplanted into B6D2F1 but not into B6 recipients attesting the efficacy of this proliferation assessment during GVHD. Loss of CFSE staining of B6 donor cells still occurred in R848-treated GVHD mice but the CFSE initial peak remained higher than in control GVHD, indicating that donor cell division was not abrogated although diminished by R848 (Fig.4A). The slower expansion of donor CD4 T cells after R848
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treatment was confirmed by the decrease in implanted donor CD4 and CD8 T cells 14 days after transplantation (Fig.4B). However, ultimately R848 did not abrogate B6 donor cell engraftment as chimerism was still detected after up to 100 days (see Fig.1G). Donor CD8 T cells were also five times less abundant in R848 than in control ncGVHD. These effects were not the consequence of R848 toxicity for T cells since host CD4 T cells were protected from the more than 50 % drop consecutive to B6 transplantation in non-treated recipient mice (Fig.4B).

The persistence of donor T cells in the R848-protected mice raised the question of their activation state. We evaluated the proportion of naïve and memory cells with CD62L and CD44 labeling respectively. The massive loss of CD62L expression by CD4 T cells observed in control ncGVHD was partially inhibited by R848 (67 % in control mice, 3.9 % in control GVHD and 26 % in R848 GVHD) and the same trend was seen for CD8 T cells, indicating that donor T cell activation was partially inhibited by R848 (Fig.4 C). In contrast, the upregulation of CD44 on CD4 T cells was not significantly modified by R848 (2.5-fold upregulation in control and 2.45 fold with R848, Fig.4 C). As to activation marker CD69, its expression was unchanged by R848 on CD8 T cells and even somewhat enhanced on CD4 T cells (Fig.4D). In contrast to B6 donor cells, there was no significant change in CD44/CD62L T cell proportions in control and R848 recipient groups. However, in control GVHD, as the B6D2F1 splenocytes were destroyed, their numbers dramatically dropped (Suppl. Fig. 1A) whereas in the R848 group, the numbers of CD44+ and CD62L- T cells were significantly more important than in control GVHD but remained in the same order than in naïve mice. In addition, an increase was observed for CD69+ CD4 T cells in R848 protected mice as compared to control B6D2F1 mice (Suppl. Fig. 1B).
We next examined the influence of R848 treatment on Tregs in the same experiments. First, we observed a ten fold decrease in the proportion of donor and recipient Foxp3+ CD4 T cells 14 days after B6 spleen cell transplantation. In contrast, in R848-treated GVHD mice, recipient Foxp3+ CD4 T cells increased four-fold while donor Tregs returned to normal B6 levels (Fig. 4E).

Together, these data indicate that during ncGVHD R848 affected donor CD4 and CD8 T cell implantation and activation but the inhibition was only partial and did not prevent the establishment of permanent chimerism. On the other hand, R848 upregulated donor and recipient Tregs.

**Tregs contribute to R848-mediated ncGVHD prevention**

The contribution of Tregs to the protective effect of R848 was tested by depletion of Tregs using anti-CD25 PC61 Ab treatment of B6 donors and B6D2F1 recipients. PC61 Ab was injected 4 days before R848 treatment and a second injection was administered one day after B6 spleen cell transplantation. As compared to donor CD4 T cells from R848 GVHD mice, B6 CD4 T cells from the PC61-R848 GVHD group engrafted host spleen faster and their numbers were significantly higher at 14 and 20 days after B6 cell transfer and continued to expand up to day 50. PC61 Ab completely depleted Foxp3+ Treg population was totally absent during the course of GVHD in contrast to R848 GVHD mice, where Tregs expanded more than six times from day 14 to day 50 (Fig. 5A). Regarding host Tregs, in PC61-R848-treated mice, half of the initial Treg population recovered 14 days after GVHD induction. On the contrary, in R848 GVHD mice, B6D2F1 Treg numbers almost doubled compared to normal B6D2F1 mice and increased four times.
Results

versus PC61-R848 ncGVHD mice and their levels remained unchanged up to day 50 after transplantation (Suppl. Fig. 2A and B).

PC61 treatment of the R848 GVHD group resulted in weight loss starting from day 17, a few days later than control GVHD mice. The percentage weight loss was finally the same in both groups, suggesting a significant contribution of Tregs in the prevention of ncGVHD morbidity by R848 (Fig. 5B). However, PC61 treatment only partially decreased survival of R848-treated mice (70% vs 90%) (Fig.5C). This trend was observed in two additional experiments.

In order to test if Treg depletion affected the level of donor T cell activation, we evaluated CD44 and CD69 expression levels 14 and 20 days after ncGVHD induction. When Tregs were depleted in R848-treated mice, CD44⁺ and CD69⁺ B6 CD4 and CD8 T cells were significantly increased and CD69 levels even exceeded those of the control ncGVHD group. As compared to day 14, the B6 CD69⁺ T cell population tripled at day 20, indicating that an absence of Tregs increased expansion of memory and activated donor T cells (Fig. 5D). However, Treg depletion by PC61 did not seem to influence early cytokine production since no significant differences in IFNγ, IL-27p28 and active TGF-β1 plasma concentrations were observed between R848- and PC61-R848-treated mice (Fig. 5E).

Together, the data suggest that Tregs from donors and recipients contributed to R848 mediated GVHD prevention. However, despite the depleting treatment, a small population of host Treg remained present, which could explain why R848 protection was not completely abrogated and resulted in death of only 30% of PC61-R848 treated mice. As shown previously, R848 GVHD protection correlates with a strong drop in pro-inflammatory cytokines and this was still observed after Treg depleting.
Results

which could also explain why R848 protective effect was not completely suppressed by Treg depletion.

R848 cooperates with anti-IL-27 Ab for modulation of acute GVHD in conditioned models.

B6 spleen cell transfer into ncB6D2F1 recipients is optimal to dissect the mechanisms involved in immune-mediated hematopoietic cell destruction and can be used as a model of induced bone marrow failure (20) but does not fully replicate allogeneic HCT. To test R848 in full allogeneic HCT, BALB/c mice were injected with R848 24h before irradiation (8Gy) and immediately after B6 spleen and bone marrow cell transfer (B6→8Gy-BALB/c). This timing aimed to avoid the requirement of donor R848 treatment that was needed for optimal protection in the B6→ncB6D2F1 model. All untreated mice rapidly developed acute GVHD and started to die on day 7 whereas 90% of the mice injected with R848 survived more than 40 days (Fig. 6A). However, R848 did not completely prevent morbidity since mice started to lose weight at day 19 (Fig. 6B). This incomplete protection by R848 was also observed in sublethally irradiated (5Gy) B6D2F1 recipients transplanted with B6 spleen cells where R848-treated mice were no longer protected against weight loss (Fig. 6C) contrary to the total protection seen in non-irradiated recipients. In these conditioned mice, R848 solely delayed disease and no longer increased survival significantly (Fig. 6C). This correlated with the persistence of pro-inflammatory cytokines IL-27p28 and IFNγ that R848 treatment failed to decrease in B6→8Gy-BALB/c (Fig. 6D) and in B6→5Gy-B6D2F1 GVHD (data not shown). As IL-27p28 was still present in the irradiated model, we tested the effect of
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anti-IL-27p28 mAb MM27.7B1 (aIL-27) combined with R848. Although this Ab completely prevents disease in the B6→ncB6D2F1 model (14), aIL-27 only partially protected in the 5Gy-B6D2F1 recipients (Fig. 6C) in agreement with data reported in a fully allogeneic transplantation model (15). Nevertheless, combining R848 and aIL-27 mAb improved survival to 100 % (Fig.6C).

In B6→8Gy-BALB/c GVHD, aIL-27-R848 combination significantly decreased IFNγ in mouse plasma although, when used separately, they did not (Fig. 6D). Moreover, the combination did not prevent T cell engraftment since we observed the same numbers of CD4 and CD8 T cells (Fig. 7A) and total B6 donor cell implantation (Fig. 7B). Already after 6 days, the number of B6 CD4 T cells recovered from the BALB/c spleen was equivalent to the total number injected and for CD8 T cells the number of cells had already increased ± 10-fold. These numbers were not modified by R848 neither by aIL-27 nor by the aIL-27-R848 combination (Fig.7A). Similarly, upregulation of memory marker CD44 and activation marker CD69 in B6 CD4 and CD8 T cells recovered 6 days after transplantation were not modified by R848. CD62L naive cell marker was completely lost in B6 CD4 and CD8 T cells recovered from control GVHD mice but remained significantly higher in the aIL-27-R848 group (Suppl.Fig.3A)

As we previously showed a Treg contribution in the R848 protection in B6→ncB6D2F1 GVHD, Treg populations were also analyzed 6 days after B6→8Gy-BALB/c GVHD induction. In the R848-treated group, Foxp3+ CD4 cells were significantly higher than in the control group and adding aIL-27 Ab to the R848 treatment further increased the Treg population 2 fold (Fig.7C). 65% of these Foxp3+ CD4 cells were positive for latent TGF-β1 associated protein (LAP), attesting to their activation state (Fig.7D). The stimulating
effect of the aIL-27-R848 combination on Foxp3-expression was even more striking for CD8 Foxp3+ T cells that were barely detectable in the donor B6 spleen but reached levels equivalent to their CD4 counterparts in the aIL-27-R848 group. This was not seen in mice treated with R848 or aIL-27 Ab only (Fig. 7C). The same result was observed in the B6→5Gy-B6D2F1 GVHD model. The aIL-27-R848 combination improved T cell implantation with a significant increase for the CD8 T cells compared to control GVHD (Suppl.Fig.3B). R848 as well as aIL-27 Ab single treatments doubled Foxp3+ CD4 T cell population of which approximately 75% were activated (Suppl.Fig. 4B) whereas the population increased almost 5 times and 85% were LAP+ in the aIL-27-R848 combination. However, in this irradiated model, the Foxp3+ CD8 population, although present, was substantially less abundant.

Thus, R848 treatment failed to completely protect against conditioned GVHD models. This protection was restored when aIL-27 treatment was added. The combination was necessary to abrogate IFNγ production and to induce maximal Foxp3+ CD4 and CD8 cell responses.

Discussion

Innate pattern recognition receptors, including TLR, are implicated in the control of GVHD (reviewed in (39)). Most often TLR stimulation aggravates disease as reported for TLR4 (40), TLR9 (41). However, the consequences may differ depending on the time of TLR stimulation with respect to aHCT as reported for TLR7/8 agonist 3M-011 (42, 43).

The present report addressed the therapeutic potential of mouse TLR7 activation by resiquimod/R848 in various GVHD models and further
improvement of efficacy by inhibition of IL-27, which we and others recently identified as a novel therapeutic target for GVHD prevention (14, 15, 44). Our results indicate that R848 administration to recipient 48h before and at the time of aHCT promoted survival in lethally irradiated recipients of fully allogeneic hematopoietic cells and in the lethal GVHD induced in ncB6D2F1 recipients of B6 parental spleen cells. R848 inhibited IL-27 production in this model but not during conditioned GVHD, suggesting that inhibition of IL-27 was not its only mode of action. This was confirmed when recipients were sublethally irradiated in the B6→B6D2F1 model. Only 5Gy irradiation almost completely abrogated the protective effect of R848 and partially that of anti-IL-27 Abs. However, combining both treatments conferred complete protection, suggesting non-overlapping actions of anti-IL-27 Abs and R848.

In the B6→ncB6D2F1 GVHD model, complete protection required treatment of donor and recipient. As the contribution of F1 recipients to GVHD is limited to antigen presentation, these results suggested that R848 impaired both APCs and T cell responders. Analysis performed just before GVHD induction confirmed this hypothesis since DC antigen presentation and T cell allore sponsiveness were inhibited when spleen cells were collected from R848 treated mice. In agreement with the literature (45), we observed that R848 induced a transient loss and functional inhibition of splenic CD11c+ DCs (both CD11b+ and CD8α+), the main cells able to induce allogeneic responses in vitro and that are known to play an important role in GVHD induction in vivo (46-48). This drop in splenic DC was very transient and antigen presentation returned to normal after 7 days (unpublished observation), suggesting that a short inactivation of host DCs is sufficient to alter the initiation of GVHD. R848 induced a similar inhibition of the capacity of T cells to respond to allogeneic in vitro stimulation. Type I IFNs seem to be
critical in the suppression by R848 of DC and T cell allo-responsiveness as both remained unaltered in R848-treated IFNAR-1−/− mice. This observation is in line with reported inhibition of DC and CD4 T cells by type I IFNs (21).

Importantly, the inhibition of T cell alloresponsiveness by R848 in vivo treatment, demonstrated by ex vivo MLC, did not prevent their implantation as chimerism was maintained for months. Moreover, the implanted T cells completely lost naive T cell marker CD62L and showed only partial inhibition of CD44 and CD69 memory and activation marker upregulations. This implied the existence of other regulatory mechanisms permitting the persistence of donor T cells in the host with reduced GVHD manifestations. A likely explanation was the effect of R848 on donor and recipient Foxp3+ CD4 Tregs. These cells dropped dramatically during GVHD but not in R848-treated mice where their numbers even increased compared to basal control levels. This was particularly striking for Foxp3+ CD8 T cells. Moreover, the presence of LAP on their surface demonstrated that these cells were in an activated state. These results are in agreement with the upregulation of Tregs by R848 reported in an asthma model (31). Given the implication of Tregs in the control of GVHD (16, 17), this Treg stimulation probably contributed to the protective effect of R848. This conclusion was substantiated by the observation that depletion of Tregs by anti-CD25 mAb treatment increased morbidity and partly impaired survival of R848-treated mice. These results are in line with GVHD impairment by in vitro R848 stimulation of Tregs (49).

R848 upregulated active TGF-β1 plasma levels during GVHD which probably contributed to the observed increase in Tregs and to the inhibition of allogeneic T cell responsiveness that characterized T cells recovered from R848-treated mice. These results are in agreement with the reported
severity of mouse GVHD induced by donors lacking SMAD3 (37) as well as the predictive impact of TGF-β expression in human aHCT donors on GVHD occurrence (50). The beneficial effect of high TGF-β1 levels could be related not only to Foxp3+ Treg cell expansion but also to the capacity of TGF-β to inhibit cytotoxic T cell development (51).

The complete and long-lasting donor Treg elimination by anti-CD25 Ab only partly suppressed GVHD prevention by R848. This phenomenon could be explained by the presence of a small host Treg population, which recovered after 14 days and remained stable during the GVHD course. This partial suppression of R848 protection could also be explained by the other suppressive mechanisms induced by the drug such as the type I IFN-mediated inhibition of DCs and T cells mentioned earlier.

An additional mechanism controlling GVHD is indoleamine 2,3-dioxygenase (IDO) (43) which was reported to be induced by TLR7/8 stimulation (52). However, a pilot GVHD experiment performed with B6 and BALB/c mice deficient for IDO1 showed that this pathway was not critical for R848-mediated protection (unpublished observation).

A final mechanism likely implicated in the prevention of GVHD by R848 was the decrease of inflammatory cytokines as IFNγ, TNFα and IL-27 in the B6→ncB6D2F1 GVHD model. In sublethally irradiated B6D2F1 mice transplanted with B6 spleen cells, R848 failed to inhibit IL-27 production and its protective effect was almost completely lost. However, combining R848 with anti-IL-27 mAb, which by itself only partly increased survival, conferred complete and permanent protection. Together, these results indicate that R848 protective activity involves but is not limited to IL-27 inhibition.

In summary, our results demonstrate that, in the context of aHCT, R848 both
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alters \textit{ex vivo} alloantigen presentation by DCs and T cell responsiveness in a process depending on type I IFN and correlating with increased active TGF-β1 as well as Treg expansion and diminished IFNγ, TNFα and IL-27 production. When not sufficient on its own, R848 protection can be further enhanced by anti-IL-27 Ab. A remarkable point of this novel GVHD preventive procedure is that it needs to be applied only just before and at the time of aHCT and results in permanent coexistence of the host and allogeneic T cells with a significant reduction of GVHD symptoms.

Acknowledgments

This work was supported by the Joseph Maisin Fund and the Interuniversity Attraction Pole of the Belgian Federal Science Policy. M.G. is FNRS-FRIA PhD Fellow at the Universite Catholique de Louvain. R.G.M. is a Haas-Teichen fellow of the de Duve Institute. JPC is FNRS research director. The technical help of Pamela Cheou and Emilie Hendrickx is gratefully acknowledged.

Author Contributions

MG designed and performed experiments, analyzed data and wrote the paper, RGM designed, performed experiments, PC designed experiments and analyzed data, CU contributed to the development of the project, LD critically read the paper and RGM, JVS, and JPC conceived the research project, designed experiments, and wrote the paper.


Results


Results

SMAD3 prevents graft-versus-host disease by restraining Th1 differentiation and granulocyte-mediated tissue damage. *Blood* 117: 1734-1744.


Results

Figure 1. R848 treatment of recipient B6D2F1 and donor B6 mice before spleen cell transfer completely blocks ncGVHD. B6D2F1 mice, treated with R848 or not (25 μg at 48 and 24 h before transplantation), were injected i.p. with 60x10⁶ spleen cells from B6 mice non-treated (NT) or treated with R848, 48 hours before cell transfer. Mice were monitored for (A) mortality and (B) weight loss. (C) R848 treatment kinetics: B6D2F1 and B6 mice were treated with R848 (25 μg) twice (6 and 5 days before; 2 and 1 days before and 5 and 6 days after B6 cells transfer) and recipients were monitored for mortality. (D) Liver sections were prepared from NT B6D2F1, control or R848 14 days-GVHD mice. H&E—stained slides were analyzed for histopathologic damage and representative sections illustrate mononuclear inflammatory cells present in portal tracts (green arrow) and large-dilated sinusoidal spaces (red arrow) are indicated. Scale bars in upper and lower panels represent 200 and 50 mm, respectively. (E) RNA was extracted from total liver and analyzed by RT-qPCR for SAA1/2 and SAA3 expression. (F) After 14 days of GVHD induction, spleen cells from control and R848 GVHD mice were recovered and stained with anti-H-2D⁰ and H-2D⁰ Abs to assess the presence of host and donor cells by flow cytometry. On the left, representative mouse dot plots illustrating percentages (gated on total living cells) and on the right, absolute numbers of host (H-2D⁰) and donor (H-2D⁰) cells are shown. Overall survivals are depicted (**p < 0.001 by Log rank test). Data are from 3 to 5 experiments (*p < 0.05, **p < 0.001 by Kruskal-Wallis test with Dunn’s Multiple Comparison Test or Anova–Bonferroni post-test).
Figure 2. R848 treatment lowers inflammatory cytokines but increases active TGFβ-1 in ncGVHD. B6D2F1 mice, treated with R848 or not (25 μg at 48 and 24 h before transplant), received 60x10⁶ spleen cells from B6 mice NT or treated with R848, 48 hours before cell transfer. (A) IFNγ, IL-27p28, active TGFβ-1 and TNFα were measured by ELISA in plasma. (B) Spleen and liver cells were analysed by RT-qPCR at day 6, 10 and 14. IFNγ gene expression normalized against β-actin is shown. RNA was extracted from total spleen and liver. Data are pooled from two independent experiments and representative of 3 (*p < 0.05, **p < 0.01, ***p < 0.001 by Kruskal-Wallis test with Dunn’s Multiple Comparison Test).
Figure 3. In vivo R848 mouse treatment affects responder and presenting cells in MLC: role of IFNAR-1. (A) B6D2F1 and B6 mice were treated or not with R848 (25 µg) 48 and 18 h before MLC of B6 responders and irradiated B6D2F1 APC. After 48 hours, (left) proliferation and (right) IFNγ production were determined by 3H-thymidine incorporation and ELISA, respectively. (B) Spleen cells from 129/Sv and 129/Sv IFNAR-1−/− mice were collected 48 hours after R848 in vivo treatment and incubated with B6D2F1 APC. Proliferations and IFNγ were measured. (C) Spleen cells were stained for CD4, LIVEST/DEAD® and Foxp3 to determine the percentage and absolute numbers of Tregs. (D) Tregs were depleted with PC61 Ab in B6 mice 4 days before R848 treatment. B6 spleen cells were collected 48 hours after R848 in vivo treatment and incubated with B6D2F1 APC and IFNγ was measured after 72 hours. (E) FVB (H2b) splenocytes were incubated without APC or with CD11b+ cDCs, CD8α+ cDCs or pDCs purified by MACS beads and FACS sorting from normal and R848-treated 129/Sv mice. After 48 hours, proliferation was recorded. (F) Spleen cells from 129/Sv and 129/Sv IFNAR-1−/− mice were collected 48 hours after R848 treatment and co-cultured with FVB responder splenocytes. Proliferations and IFNγ were measured. Data are from 2 to 4 experiments in all panels (*p < 0.05, **p < 0.001 by Kruskal-Wallis test with Dunn's Multiple Comparison Test).
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Figure 4. R848 treatment lowers effector T cells but increases Tregs during ncGVHD. (A) B6D2F1 and B6 mice, treated with R848 or not, received 15×10⁶ B6 CD5⁺ cells labeled with CFSE from B6 mice NT or treated with R848 48 hours before cell transfer. After 6 days, anti-H-2D⁺⁻, -H-2Dk and -TCRβ Abs were used to analyse the proliferation level of B6 T cells in host spleen by flow cytometry. (B-E) 14 days after total B6 spleen cell transfer, spleen cell subsets were enumerated by FACS, using anti-H2D⁺⁻, -H-2Dk, -CD4, -CD8, -CD44, -CD62L and -CD69 Abs. (B) Plots show absolute numbers of CD4 and CD8 T cells. (C) CD44, CD62L and (D) CD69 expression by T cells are represented with density plots or histograms for percentage and plots for absolute numbers. (E) H-2D⁺⁻, H-2Dk, LIVE/DEAD®, CD4, TCRβ and Foxp3 staining was used to determine by flow cytometry the percentage (left) and absolute number (right) of Tregs in host spleen 14 days after GVHD induction. Data are from 2 to 3 experiments in all panels (*p < 0.05, **p < 0.01, ***p < 0.001 by Kruskal-Wallis test with Dunn’s Multiple Comparison Test or Mann-Whitney unpaired t-test).
Figure 5. Foxp3+ Tregs are involved in GVHD prevention by R848. Tregs were depleted with PC61 Ab in donor and recipient mice 4 days before R848 treatment and 1 day after GVHD induction. (A) After 14, 20 and 50 days of B6 cell implantation, H-2D\(^\beta\), H-2D\(^k\), LIVE/DEAD*, CD4, TCR\(\beta\) and Foxp3 staining was used to evaluate by flow cytometry Treg populations. Mice were monitored for (B) weight loss and (C) mortality. (D) anti-CD44 and -CD69 Abs were used to analyze T cell activation after 14 and 20 days. (E) IFN\(\gamma\), IL-27p28 and active TGF\(\beta-1\) were measured by ELISA in plasma prepared from mice bled on day 6, 14 and 20. Data are from 2 to 3 experiments in all panels (*p < 0.05, **p < 0.01, ***p < 0.001 by Kruskal-Wallis test with Dunn's Multiple Comparison Test, Mann-Whitney unpaired t-test and Anova-Bonferoni post-test).
Figure 6. R848 and all-27 Ab administration to recipient mice prevents aGVHD in irradiated models. (A-B) BALB/c recipient mice were irradiated with 8 Gy and treated or not with R848 (25 μg/mouse) 48 and 0 h before transplantation of 2x10^6 B6 CD5+ splenocytes and 10^7 B6 TCD-BM cells. Mice were monitored for (A) survival and (B) weight loss. (C) B6D2F1 recipient mice were irradiated with 5 Gy and treated or not with R848 (25 μg/mouse) 48 and 24 h before transplantation of 4x10^5 splenocytes and 10^7 TCD-BM cells from B6 mice. In addition, both recipient groups were treated or not with all-27 (0.5 mg) at days 0 and 6. Mice were monitored for survival and weight loss. Data are pooled from two experiments and representative of three (**P < 0.01, ***P < 0.001) Log rank test. #p < 0.05, ##p < 0.01 and ###p < 0.001 for significance between Control GVHD and all-27 GVHD, $p < 0.05 and $$$p < 0.01 for significance between Control GVHD and R848 GVHD and *p < 0.05 and ***p < 0.001 for significance between Control GVHD and all-27-R848 GVHD determined by a two-way ANOVA with Bonferroni post-test). (D) After 6 days of B6 cell transfer, IFNγ and IL-27p28 were measured by ELISA in plasma from irradiated-GVHD BALB/c mice. Data are representative of 2 experiments. (*P < 0.05, **P < 0.01, ***P < 0.001 by Kruskal-Wallis test with Dunn's Multiple Comparison Test).
Figure 7. all-27-R848 combination induces strong Treg activation in aGVHD in irradiated recipients. BALB/c recipient mice were irradiated with 8 Gy and treated or not with R848 (25 μg/mouse) 48 and 0h before transplantation of 2×10⁶ CD5⁺ splenocytes from B6 mice. In addition, recipients were treated or not with a-IL-27p28 at days 0 and 6. After (A) 6 and (B) 40 days of GVHD induction, spleen cells were recovered and stained with anti-H-2D⁺, -H-2D⁻, -CD4 and -CD8 for FACS spleen cell subset analysis. (D-C) H-2D⁺, H-2D⁻, LIVE/DEAD*, CD4, CD8, LAP and Foxp3 staining was added on 6-day-GVHD spleen cells to evaluate Treg populations. Data are from 2 to 3 experiments (**p < 0.01, ***p < 0.001 by Kruskal-Wallis test with Dunn’s Multiple Comparison Test).
Supplementary Figure 1. Modulation of B6D2F1 T cell populations in control and R848 ncGVHD. B6D2F1 mice, treated with R848 or not (25 μg at 48 and 24 h before transplantation), received 60x10^6 spleen cells from B6 mice non-treated or treated with R848, 48 hours before cell transfer. 14 days after B6 spleen cell transplantation, spleen cell subsets were enumerated by FACS, using anti-H-2D^b, H-2D^d, -CD4, -CD8, (A) -CD44, -CD62L and (B) -CD69 Abs. The levels of T cell activation are represented with density plots for percentage and scatter plots for absolute numbers. Data are representative of two independent experiments (***p < 0.01 by Mann–Whitney unpaired t-test).
Supplementary Figure 2. PC61 treatment strongly reduces Treg number in R848-GVHD mice. Tregs were depleted with PC61 Abs in donor and host mice 4 days before R848 treatment and 1 day after GVHD induction. H-2D\(^b\), H-2D\(^k\), LIVE/DEAD\(^\ast\), CD4, TCR\(\beta\) and Foxp3 staining was used to evaluate by flow cytometry Treg population 14, 20 and 50 days after B6 cell transfer. Data are representative of 3 independent experiments (\(^*\)p < 0.05, \(^{**}\)p < 0.01 Mann–Whitney unpaired t-test).
Supplementary Figure 3. all-27-R848 combination prevents aGVHD in irradiated models. (A) BALB/c recipient mice were irradiated with 8 Gy and treated or not with R848 (25 μg/mouse) 48 and 0 h before transplantation of 2 x 10⁶ splenocytes from B6 mice. In addition, recipients were treated or not with all-27 (0.5) at days 0 and 6. After 6 days of GVHD induction, spleen cells from control GVHD and R848-MM27 GVHD mice were recovered and stained with anti-H-2D, -H-2D, -CD4, -CD8, -CD69, -CD44 and -CD62L Abs for FACS spleen cell subset analyses. (B) B6D2F1 recipient mice were irradiated with 5 Gy and treated or not with R848 (25 μg/mouse) 48 and 0 h before transplantation of 4 x 10⁵ B6 splenocytes. In addition, both recipient groups were treated or not with all-27 (0.5 mg) at days 0 and 6. After 7 days of GVHD induction, spleen cells were recovered and stained with anti-H-2D, -H-2D, LIVE/DEAD®, anti-CD4, -CD8, -LAP and -Foxp3 Abs to evaluate Treg populations. Data are representative of 2 experiments (*p < 0.05, **p < 0.01, ***p < 0.001 by Kruskal-Wallis test with Dunn’s Multiple Comparison Test).
Discussion

1. TLR7 activation by LDV / R848 protects mice against acute GVHD

Several studies have shown the involvement of TLRs in the GVHD pathophysiology. Dependent on the TRL activated, disease development can be hampered or amplified [57].

In our studies, we have demonstrated the inhibitory effect of TLR7 activation in aGVHD mouse models. TLR7 stimulation by LDV or R848 abrogated GVHD induced by transfer of B6 spleen cells to B6D2F1 recipients. This protection, was associated with prevention of liver damage and a complete suppression of serum amyloid A (SAA)1/2 and SAA3 expression. TLR7 activation led to permanent splenocyte chimerism without destruction of host cells. In addition, TLR7 activation led to an inhibition of Th1 cytokines such as IFNg and IL-27 and up-regulation of active TGF-ß1 and Foxp3+ Tregs.

These observations seem to be counterintuitive since LDV and R848 are known to stimulate Th1 responses [222, 247].

It has been already highlighted that LDV infection can affect the course of autoimmune diseases, probably via modulation of the immune response. For instance, in a lupus model, LDV infection leads to a decrease in the production of antibodies directed against nuclear antigen and red blood cells. Moreover, in susceptible mice which develop a high amount of autoantibodies and a severe immune complex glomerulonephritis with autoimmune haemolytic anemia, LDV infection decreases significantly the amount of antibody synthesized and the mortality [227]. This is also the case in an experimental allergic encephalomyelitis (EAE) model where LDV suppresses disease
dev
elopment [230]. However, the mechanisms underlying these protective effects remain unknown.

R848 administration can also lead to immune regulation. In an induced experimental model of murine allergic asthma, this TLR7 ligand increases Treg numbers in the lungs and this effect is mediated through a TGF-β-dependent pathway [254].

Together, these observations suggest that the immune response triggered by TLR7 stimulation depends on the context. In resting mice, LDV and R848 promote a Th1 response. However, in some disease environments, TLR7 activation seems to stimulate regulatory responses and decreases the pathology.

The timing of the TLR7 stimulus should also be taken into consideration. Indeed, we have shown that mice treated with R848 48h and 24h before the allo-HCT survive and do not develop GVHD. However, if the R848 treatment is performed 6 days before allo-transfer or 6 days after it, recipient mice were not protected and developed lethal GVHD, indicating that the timing of TLR7 activation is important to confer a protection against GVHD. This phenomenon has already been reported with another TLR7 ligand, 3M-001. Repetitive applications of 3M-001 after allo-HCT aggravated GVHD severity while a single treatment between TBI and allo-HCT induced expression of the immunoinhibitory enzyme indoleamine 2,3-dioxygenase (IDO) in host APCs, which resulted in reduced lethal intestinal GVHD [74, 80]. This administration timing, just before allo-transfer, seems to be really important to hamper GVHD initiation.

Incidentally, a pilot GVHD experiment that we performed with B6 and BALB/c mice deficient for IDO1 showed that this pathway was not critical for R848-
mediated protection. A similar observation has been reported by the Gorski's study showing that despite of 3M-001 and R848 are both imidazoquinoline, they activated differently NK in human [272]. In addition, it was also described that 3M-001 was shown to selectively activate human pDC whereas R848 induced cytokine production from both human pDC and mDC [234]. Together, these observations suggested that the generated immune response depended on the TLR7 agonists.

Another explanation regarding the inhibitory effect of TLR7 stimulation might be that an early T\textsubscript{H}1 differentiation disrupts the pathophysiology development of GVHD. It is well established that a T\textsubscript{H}1 response is crucial for the GVHD development [105-107, 273]. Nevertheless, several studies have reported that mice treated by IFN\textgamma or IL-12 just before or during allo-HCT develop a less severe aGVHD [114, 115]. This notion can be linked with the idea that treatment timing seems crucial to avoid GVHD.

The importance of T\textsubscript{H}1 response in GVHD has been also reported by Marillier and colleagues. In a parent to F1 mouse model, the blockade of IL-27p28 strongly decreases IFN\textgamma and increases Treg population resulting in prevention of aGVHD [1]. We have confirmed these results in another mouse GVHD model (B6 donor to lethally irradiated BALB/c). This phenomenon suggests that the modulation of T\textsubscript{H}1 response may impair the GVHD initiation and lead to a regulatory process.

Finally, it would be interesting to test R848 treatment on GVL model. This potential anti-tumor immunity has been clearly identified in mouse models of lymphoma or CT26 solid mouse tumor [251, 252]. However, based on its complex properties, it is difficult to predict its effect against tumor cells in a GVHD context.
2. What are the mechanisms involved in R848-GVHD protection?

LDV-infected and R848-treated mice show a transient elimination of splenic CD11b+ and CD8α+ cDCs required for allogeneic CD4 and CD8 T cell responses in vitro. A second effect of TLR7 stimulation is a decreased T cell proliferation and IFNγ production during MLC. Both cDC and responder T cell inhibition are type I IFN-dependent.

Type I IFNs were first described as cytokines that stimulate the immune response against viral infections and confer cell resistance to viruses [274]. However, it is now established that type I IFNs play an important role in orchestrating both innate and adaptive immunity. After TLR7 stimulation, type I IFNs are secreted by pDCs and activate CD4 T cells resulting in the upregulation of the CD69 activation marker [220]. In addition, multiple studies have investigated the effects of type I IFN in anti-tumour response and globally these cytokines are able to activate NK cells as well as maintaining CD8 T-cell responses against tumor [102]. On the other hand, type I IFNs can induce immune suppressive effects. For instance, LDV infection is known to enhance the susceptibility of the host to endotoxin shock. Indeed, LDV-infected mice deficient for IFNAR-1 develop more severe septic shock than wild-type counterparts [222]. Another study has also shown a protective effect of type I IFNs on aGVHD. Mice deficient for IFNAR-1 transplanted with donor T cells developed a more severe aGVHD than wild-type recipients. This result was corroborated with a decreased gastro-intestinal GVHD intensity in recipient mice treated with IFNα one day after allo-transplant [275].

In our study, we have shown that LDV infection induces a sharp peak of type I IFNs at the time of allo-HCT. This implies that the donor cells were only shortly exposed to an IFN-rich environment but this was sufficient to impair their allogeneic response. Regarding R848 treatment, we were unable to
detect type I IFN in mouse plasma. Nevertheless, we have shown that R848 induces a strong increase of CD69+ T cells, despite their hypo-responsiveness that was mainly dependent on IFNAR-1 (unpublished results). This early activation state of T cells could disturb their reactivity against allo antigen leading to hypo-responsiveness in MLC. This impaired T cell allo-responsiveness induced by R848 after 48 hours, did not involve Tregs.

Taken together, type I IFNs induce an inhibitory effect on allo-T cells and cDCs in response to TLR7 stimulation and this correlates with GVHD prevention.

Following these observations, we have attempted to discover which cell subset is involved in TLR7 effects. The main cell populations that express TLR7 in mouse are pDC and B cells [233, 238]. Elimination of pDCs with 120G8 depleting antibody did not affect LDV and R848 protective effects (unpublished results).

Recently, we have tested the R848 treatment in mice deficient for B cells (B6-Mu−/−). When B6 splenocytes were treated in vitro with R848 for 48h, T cells were unable to react against allo-APC in MLC whereas T cells from Mu−/− mice were not affected by R848 and proliferated normally in response to Allo-APCs (Figure 5 A). To confirm the B cell involvement, CD4+ and CD19+ (B cells) splenocytes were purified and cultured together or separately for 48h with R848. After pre-culture, cells were washed and stimulated on fresh Allo-APCs. When CD4 T cells were cultured with CD19 cells and R848, their proliferation was strongly reduced compared to untreated cells or CD4+ cells treated alone. The same experiment was performed with CD8 T cells. However, they were not affected by the R848 treatment and the presence of B cells (Figure 5 B). These preliminary results indicated that R848 acts on B cells, which suppress directly CD4+ T cells. However, for CD8 T cells the results seem to indicate that B cells act indirectly on CD8 T cells.
A B cell suppressive effect has already been observed in GVHD. Rowe and colleagues have described that recipient mice deficient for B cells have an exacerbated GVHD and that this inhibitory mechanism of B cells is IL-10 dependent [276]. Moreover, the tolerogenic effect of B cells has been already described in several experimental models. The regulatory B cells (Breg), via IL-10, lead to autoimmune suppression [277]. B cells were reported to stimulate Treg expansion by TGF-β in an allograft model [278]. On the other hand, B cells are able to limit T cell differentiation via PD-L1 contact as shown in EAE model [279].

The tolerogenic effect of B cells opens several perspectives and it would, therefore, be interesting to test those cells in GVHD models and see if their suppressive effect is sufficient to abrogate the disease.
3. Improvement of R848 treatment in a clinical perspective

a. In vitro R848 treatment to avoid R848 administration into patients

As mentioned in the introduction, in man TLR7 is expressed in many hematopoietic cell types as well as in organ tissues such as bronchial cells [280] whereas, in the mouse, it is mainly restricted to pDC and B cells. In the mouse GVHD model, R848 treatment is therefore really specific to two target populations. However, systemic R848 treatment in human patients may lead to an activation of all populations with the risk of inducing side-effects. In addition, several investigations have reported the presence of TLR7 in tumor cells. Cherfils-Vicini and colleagues have demonstrated the expression of TLR7 in tumor cells in human lung cancer and their stimulation with TLR7 agonists led to an increased tumor cell survival, and chemoresistance [280]. This is also the case in human pancreatic cancer, where cancer cells TLR7 expression increases tumor cell proliferation and promotes chemoresistance [281]. Together, these data emphasize that TLR signaling can directly favor development of certain tumors. Therefore, it seems risky to elaborate a human R848 prophylactic treatment against GVHD in patients with malignant disorders.

An alternative to avoid R848 administration into the patient would be to treat donor cells in vitro prior to the allo-transfer. In order to investigate whether R848 in vitro treatment could have a clinical impact, we tested its effect in a full allogeneic mouse GVHD model (B6 donor to lethally irradiated BALB/c). Figure 6B illustrates that R848 in vitro treatment is also able to prevent aGVHD. To verify if the graft was not rejected, spleen chimerism (H-2D<sup>e</sup>/H-2D<sup>b</sup>) was analyzed 40 days after transplant by flow cytometry. B6 cells were
able to engraft in BALB/c spleens since the majority of BALB/c splenocytes were H-2D\textsuperscript{b} cells (Figure 6C).

These preliminary results lead to more clinically relevant perspectives since R848 treatment is performed \textit{in vitro} with donor splenocytes.

\textbf{b. Cooperation between all-27 and R848 enhances the protection in severe GVHD}

Tregs are a subset of peripheral CD4\textsuperscript{+} T cells whose function is to maintain immune tolerance in healthy individuals [282, 283]. Reduction of Tregs is associated with loss of immune tolerance and development of autoimmunity.
Discussion

Rezvani and colleagues have reported that donor Treg level is predictive of aGVHD. A high Treg content is associated with a lower risk of GVHD in HLA-matched patients [200]. Several clinical studies have also shown that infusion of native donor Tregs is correlated with a lower incidence of aGVHD and an improvement of immune reconstitution [197-199]. However, one potential limiting factor to use this therapy of adoptive Treg transfer is the complex method to produce sufficient numbers of Tregs for infusion without any effector T cell contamination [284].

In our studies, we have shown in a parent to F1 non-irradiated model that R848 administration decreased effector T cell reaction and increased donor and recipient Tregs during aGVHD. These Treg populations were crucial to prevent the disease development and their depletion with PC61 antibodies hampered protective effect of R848 treatment. This increase of Treg population was also observed with aIL-27 blocking antibody. However, if the mouse GVHD model was a full allogeneic model (B6 to lethally irradiated BALB/c mice), the aIL-27 Ab treatment only partially protected against this lethal GVHD. The same results were observed with R848 treatment which failed to completely protect against conditioned GVHD models. Interestingly, when R848 administration was combined with aIL-27 Ab treatment, we restored a complete protection in conditioned GVHD models. This combination was necessary to abrogate completely the production of inflammatory cytokines and to induce a strong Foxp3+ CD4 and CD8 cell response, which was almost twice that obtained with single treatments (R848 or aIL-27 Ab).

These findings showed that when not sufficient on its own, R848 protection can be further enhanced by anti-IL-27 Ab. Therefore, this widens the potential therapy to severe aGVHD models.
Conclusion

Acute GVHD remains the most important complication of allogeneic HCT despite the improvements in disease comprehension and treatments.

Our work has allowed to highlight immune suppressive response by TLR7 stimulation in a GVHD context. Our results have shown that TLR7 activation inhibits cDCs and allo responsiveness of T cells. Both inhibitions are dependent on type I IFN and for T cells also on B cells. This suppressive effect seems to hamper the GVHD initiation in a parent to F1 model without affecting long-lasting implantation of donor T cells. In addition, GVHD prevention is associated with a decrease of IFNγ, TNFα and IL-27 but an upregulation of active TGF-β1 plasma levels. We have also shown that donor and host Foxp3+ Treg cell numbers were increased in recipient mice and that their elimination compromised survival and exacerbated morbidity in R848-treated mice.

In conditioned aGVHD models, the aIL-27-R848 combination is required to obtain an optimal protection characterized by an upregulation of CD4 and CD8 Foxp3+ Treg levels.

Finally, we recently found that donor HSC treated in vitro with R848 leads also to donor engraftment without aGVHD development. This opens new treatment perspectives since in this context, the recipients and the donors do not undergo R848 administration.

We conclude that R848 modulates multiple aspects of GVHD and offers potential for safe allogeneic cell transplantation that can be further optimized by IL-27 inhibition.
Conclusion

Figure 7. (A) Schematic representation of aGVHD development. (B) Schematic representation of aGVHD inhibition following TLR7 stimulation.
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