B CELLS AND REGULATORY T CELLS IN GRAFT VERSUS HOST DISEASE

A CLINICOPATHOLOGICAL STUDY IN HUMANIZED MICE

Marieke Hogenes

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B CELLS AND REGULATORY T CELLS IN GRAFT VERSUS HOST DISEASE

A CLINICOPATHOLOGICAL STUDY IN HUMANIZED MICE

B-cellen en regulerende T-cellen in graft-versus-host-ziekte
een klinisch-pathologische studie in gehumaniseerde muizen

(met een samenvatting in het Nederlands)

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Marieke Cornelia Hendrika Hogenes

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Promotoren: Prof. dr. P.J. van Diest
Prof. dr. M.R. van Dijk

Copromotor: Dr. R.A. de Weger

A prudent question is one half of wisdom

Francis Bacon

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CHAPTER 1

AN INTRODUCTION TO GRAFT-VERSUS-HOST DISEASE AND IT'S PLAYERS OF INTEREST

"…So far as power and discernment shall be mine,
I will carry out regimen for the benefit of the sick,
and will keep them from harm and wrong..."

This citation from the ancient Hippocratic oath is probably one of the most challenging ambitions in today's medicine. When facing hematologic malignancies, allogeneic hematopoietic cell transplantation (HCT) is often one of the potential curative options. Unfortunately, despite its proven efficiency to cure the initial disorder, HCT is not completely safe. It may be accompanied by signs of rejection of the recipient by the grafting donor cells, resulting in acute and / or chronic graft-versus-host disease (GvHD) and this may cause not only severe morbidity, but even death of those whose life we meant to save. Risk factors for acute and chronic GvHD include not only human leukocyte antigen (HLA) mismatching [1], but also appears to be influenced by the recipients age, CMV seropositivity of both recipient as well as donor, total body irradiation and gender mismatch [2-5] .

Only two to three decades ago, GvHD was thought to be a primarily T cell-mediated disease and a line was drawn at 100 days after bone marrow transplantation (BMT) to separate acute from chronic GvHD. Removal of these T cells from the transplanted cells almost completely prevents GvHD, but it comes at a cost of increased incidences of graft rejection and relapse of original disease [6].

During the last 20 years of research however, scientists learned that the pathogenesis of this disease is far more complex than we could have ever imagined and apart from T cells, many other cells and mechanisms play an important role as well.

Current concepts of pathogenesis in acute and chronic graft-versus-host disease

In today's medicine, development of acute and chronic symptoms is no longer strictly bound to the arbitrary line of 100 days, as acute symptoms can develop after 100 days post-BMT and chronic features may start before this imaginary time limit. This consideration resulted in updated criteria to define acute and chronic GvHD by the National Institute of Health (NIH) Consensus working group in 2006 [7], with NIH updates on histological criteria in 2014 [8].

According to the most recent data, acute and chronic GvHD have different kinetics and pathogenesis [9], although in both acute as well as chronic GvHD antigen presenting cells (APC's) play a central role [10, 11].

Although a strong predictor for the development of acute GvHD remains the degree of disparity between HLA gene expression [12, 13], acute GvHD is still a major issue after fully matched-HLA transplantations. Acute GvHD is suggested to be the result of a three-step process and does not require alloantigen expression to occur [14]. First, tissue damage due to treatment and myeloablative regiments prior to transplantations results in the release

of inflammatory cytokines [15]. Second, mature donor lymphocytes from the graft respond to these cytokines with expansion and activation, especially when in contact with host- and donor APC's, in which host APC's are related to the occurrence of GvHD and donor APC's to the maximal GvHD reached [16]. As a result, in step three, these expanded activated responder T cells induce additional damage [17].

Less is known regarding the pathogenesis of chronic GvHD, although several hypotheses have been proposed. It has been suggested that chronic GvHD is more likely the result of polymorphic antigens common to host and recipient [9] and therefore, more likely to represent a form of autoimmune disorder. T cell reconstitution after transplantation is derived from mature donor T cells as well as thymic-dependent T cell maturation from donor stem cells, but there might be an issue with the latter in the damaged environment that remains after myeloablative therapies and irradiation. Maturation of T cells from donor stem cells in a damaged environment might result in an ability of pathogenic T cells to escape from central negative selection within the thymus and they might start acting as autoreactive cells [9, 18-20]. Another hypothesis is that the presence of alloreactive T cells might be related to activation of autoreactive B cells, derived from the pool of circulating memory B cells [6]. Either way, with regard to the pathogenesis of organ manifestation of chronic GvHD, there is no direct evidence to support a causative relation of autoantibodies to e.g. fibrosis [6]. Nevertheless, chronic GvHD can show histological changes very similar to autoimmune collagen diseases like scleroderma and systemic lupus erythematosus (SLE) and often an increase in collagen with less T cell infiltration compared to acute GvHD is seen [21, 22].

Regulatory T cells

In the past decade, regulatory T cells (Tregs) were discovered to be highly important in GvHD [23, 24]. These Tregs represent a subset of cells within the CD4+ T cell population (percentages varying from 1-2% to approximately 5-10% of the CD4+ population). Tregs show suppressive capacity on alloreactive T cells by expressing Forkhead protein 3 (FoxP3), which induces indoleamine 2,3 dioxygenase (IDO) in macrophages, resulting in tryptophan degradation and downregulation of T cell proliferation [25]. The outcome is a decrease in graft-versus-host response.

Two different types of Tregs can be identified: cytokine-independent Tregs and cytokine-dependent Tregs [26]. For the latter, cytokines like for example TGF-β are essential, but Il-6 (a cytokine induced by e.g. myeloma), has the capacity to inhibit its action [27]. Nowadays it is accepted that these 2 types of Tregs are in fact represented by 4 different sets of Tregs:

natural thymic derived Tregs (nTregs) requiring cell-cell contact, anergic T cells (also requiring cell-cell contact), cytokine inducible Tregs (Tr1Tregs) and Th3 Tregs, which are also cytokine dependent [28].

However, in most reports Tregs are usually simply referred to as the (CD4+CD25+)FoxP3+ T cell population and we have learned that use of *in vitro* expanded and activated Tregs usually show a better inhibiting effect on GvHD compared to freshly isolated Tregs [29].

B cells

As indicated above in the current concepts of the kinetic pathways of both acute as well as chronic GvHD, B cells should not be excluded for their importance. Their function within the reaction is however still poorly understood.

B cells appear to play a role in both acute as well as chronic GvHD, acting as APC's and producing autoantibodies [30] , and also producing other important cytokines and chemokines as well [6]. In both acute and chronic GvHD, the observed reaction is accompanied by low reconstitution of the B cell population [31].

Levels of B cell activating factor (BAFF) are usually high in the period shortly after transplantation. This cytokine is related to the reconstitution and survival of B cells. When found to be continuously high, BAFF prevents apoptosis of low-affinity autoreactive B-cells [32, 33]. GvHD has been indicated to be more related to existing mature autoreactive CD4+ T cells and B cells, rather than *de novo* derived ones [34] and a relation between GvHD and improved survival of autoreactive B cells therefore would not be surprising.

The B cell expression of CD80/CD86 as well as CD40 and the related interaction with T cells, lowers the required threshold for T cell activation. It improves both T cell differentiation as well as T cell survival [35]. Resting B cells show low expression of costimulatory molecules [6]. It's hypothesised that B cell depletion with Rituximab (an anti-CD20 pharmakon) within 6 months prior to allogeneic stem cell transplantation results in a lower risk of developing acute GvHD [30], possibly due to diminished antigen expression by B cells and a decreased activation of donor T cells [36]. Rituximab induces a correction of the aberrant expression of CD40 and CD80 in patients with autoimmune disease [37], resulting in diminished T cell activation. The most significant change in T cell subsets after Rituximab treatment (in lupus nephritis patients) is the reduction of CD4+ cells with the expression of costimulatory/ activation T cell molecule CD40L [38].

Timing of the B cell depletion however, seems crucial to get effective results without interference with engraftment [6]. Nevertheless, response to Rituximab treatment is usually partial and rarely complete. For example, chronic cutaneous GvHD as well as musculoskeletal involvement usually respond well, in contrast to chronic GvHD in visceral organs [6]. This might be related to the location-dependent speed for Rituximab activity and effectiveness: within minutes, the circulating compartment is effectively depleted of B cells (>90%), but a slower rate is seen in lymph nodes and spleen with approximately 60-70% depletion in 24 hours, while peritoneal depletion of B cells takes almost a week to be accomplished [39, 40].

Although at present many reports confirm the beneficial effects of Rituximab in GvHD patients, many of today's reports fail to include an histological analysis or include only limited histological insights on the morphological changes, e.g. cutaneous GvHD only [41-44].

B cells and Tregs in the Graft-versus-Host battlefield, friends or enemies?

Indications that B cells and Tregs might be related are numerous:

1). Some reports focus on Tregs, which are said to directly supress B cells due to their presence in both the T cell zone as well as the mantle zone of lymph follicles [45].
2). Tregs might be deficient in chronic GvHD [23] and B cell depletion results in restoring sufficient Treg levels, leading to a clinical beneficial effect on symptoms [46].
3). Often, a prominent raise in mRNA levels of genes characterizing Tregs can be noted in patients treated with Rituximab in an early phase of B cell depletion, with increased mRNA levels for CD25, FoxP3, CTLA-4, GITR and TGF-β1 [38]. Other studies support this.
4). Stasi *et al*. report that both numbers and suppressive function of Tregs increase after Rituximab treatment and increased levels of Tregs have been shown in acute Rituximab depletion in other studies as well [47, 48].

However, many studies do not take the activation status of the B cells into consideration [47]. The activation status is important though, because having an activated B cell population appears to be unfavourable for the clinical ability to respond to B cell depletion with decreased GvHD signs [49]. Activation of B cells is said to induce Treg-inhibition and -proliferation *in vitro* [30, 50]. Others oppose this by indicating that *in*active, resting B cells induce expansion of Tregs by prolonging lifespan or proliferation under influence of TGF-β3 production and suggest that after activation of the B cell, this effect on Tregs is lost and favours expansion of CD4+ effector T-cells [51].

Lower levels of Tregs in monoclonal gammopathy of unknown significance (MGUS) and myeloma, both plasma cell derived disorders, have been reported [52] but contradicting reports indicating increased levels of Tregs in both MGUS as well as myeloma also exist [53]. Some report a loss of suppressive activity of Tregs in plasma cell dyscrasias, even when additional Tregs are added, others do not show any differences [52, 54].

The answer to the contradicting findings, might be found in differences in study designs. Nevertheless, in most GvHD studies to B cells, Tregs or both, the study design focusses on peripheral blood and bone marrow; analysis of other visceral organs within the studies is usually not performed.

Aim of this thesis

This thesis focusses on B cells and regulatory T cells with respect to their role and possible interaction in graft-versus-host disease from a different perspective: histology.

A huge difference between this thesis and known literature on this topic, is that significantly more emphasis is placed on the histomorphology of this disease in *all* organs involved, rather than clinical phenotype or peripheral blood only.

In our hypothesis we consider that the interaction might not be restricted to quantitative cell numbers, but could also influence their migration to and within several visceral organs as well as their functional capacity within the organ. The addition of thorough histomorphology is considered to be a key-feature to explain current contradictions and remaining questions in literature regarding Rituximab treatment of GvHD.

Outline of this manuscript

In **Chapter 2** we will explore the possibilities of murine models for our study to GvHD. The use of a xenogeneic mouse model is very attractive, due to the ability to explore and separate donor from host responses in an immunohistochemical analysis as well as in the cytokine analysis. It might feel arbitrary to use 2 different species, although trogocytosis (a mechanism showing bidirectional transfer of surface antigens between donor and host) is not limited to humans and can be found in chimeric mouse models as well [55, 56]. Constituting a xenogeneic model for our study has several benefits for our topic of interest, as will be outlined in this chapter.

A thorough study remains necessary, to be able to properly evaluate histological changes of any kind in our experiments with a xenogeneic mouse model. **Chapter 3** defines the kinetics and morphology of the xenogeneic graft-versus-host response in our chosen RAG2$^{-/-}$ γc$^{-/-}$ mouse model after introduction of human peripheral blood mononuclear cells (huPBMCs). It provides the basic knowledge of the morphological changes in our xenogeneic mouse model, as well as the normal cytokine-profile in the induced graft-versus-host response.

In **Chapter 4**, the evaluation of the xenogeneic RAG2$^{-/-}$ γc$^{-/-}$ mouse model is continued for modifications within this model, regarding the effects of macrophage- as well as early B cell depletion.

Chapter 5 focusses on the effects of B cell activation in relation to T cell proliferation and Treg induction as well as function, combining an *in vitro study* to evaluate different activation steps of B cells with *in vivo* studies to the xenogeneic graft-versus-host pattern in the presence of myeloma.

Chapter 6 outlines the current difficulties in coherence between clinical and histological grading of cutaneous graft-versus-host disease in human and highlights that the use of histology in human GvHD is much more complicated and prone to confounders, contrasting the valuable contribution of histology in our murine model.

Chapter 7 contains a summary and general discussion, with an overall conclusion on how B cells and Tregs relate to one-another according to our findings.

In **Chapter 8** a summary is provided in Dutch.

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CHAPTER 2

HUMANIZED MOUSE MODELS IN TRANSPLANTATION RESEARCH

M.C.H. Hogenes ϕ, M.M.H. Huibers ϕ, C. Kroone , R.A. de Weger
ϕ Authors contribution equally to the work

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ABSTRACT

The interest in the use of humanized mouse models for research topics like graft-versus-host disease (GvHD), allograft studies and other studies to the human immune system is growing. The design of these models is still improving and enables even more complicated studies to these topics. For researchers it can be difficult to choose the best option from the current pool of available models. The decision will depend on which hypothesis needs to be tested, in which field of interest, and therefore 'the best model' will differ for one to another.

In this review, we provide a guide to the most common available humanized mouse models, with regards to different mouse strains, transplantation material, transplantation techniques, pre- and post-conditioning and references to advantages and disadvantages. Also, an evaluation of experiences with humanized mouse models in studies on GvHD and allograft rejection is provided.

INTRODUCTION

In many studies on human immunity and cancer, there is a growing interest for the use of (small sized) humanized animal models. The benefit is that one can test parameters of human cells directly and theoretically the results do not have to be translated from another (donor) species. However, the formation of a xeno-transplantation-situation can make the interpretation of results difficult, as it is not known whether the human cells will behave exactly the same as in a human recipient.

Although 'the perfect' mouse strain to study the human immune system is still to be found, the current available strains do give the opportunity to study parts of the human immune system in a set context. Many of these strains come with disadvantages, which might severely hamper their suitability for specific studies.

This review provides an overview of the most common currently available strains of immune compromised or -deficient mice, an outline of the human material transplants and transplantation techniques, including their benefits and restrictions with a summarizing flow chart (Figure 1). We will guide researchers along the most well-known humanized mouse models, to enable them to make a suitable choice for their studies in graft versus host disease and/or allograft (transplant) rejection.

Mouse strains

One of the first gene-mutations found resulting in severe combined immunodeficiency in mice was the *Prkdc*^{scid} mutation in a CB17-mouse strain. These mice have a loss-of-function mutation of the Prkdc gene. This gene encodes the catalytic subunit of a DNA dependent protein kinase with a role in resolving the DNA double strand breaks that occur during V(D)J recombination. In the absence of V(D)J recombination, the T cell receptor (TCR) gene in T cells and the immunoglobulin (Ig) gene in B cells are not expressed. This *Prkdc*^{scid} mutation made it impossible for these mice to produce mature T and B cells with functional surface receptors [1, 2], but first reports often showed only low level engraftment when introducing human cells in these mice [3-5]. Human T cells became anergic and/or xenospecific selection of the T cell repertoire occurred [3, 6, 7]. The CB17-*scid* mice also showed leakiness of T and B cells. Some improvement was achieved with the use of γ-irradiation [8, 9] destroying almost all murine stem- and haematopoietic cells, but the *scid* mutation results in an overall defect in DNA repair and causes more radiation sensitivity. A second injection of human cells a few days after the initial injection and chemical macrophage depletion has been suggested to improve engraftment [10-12]. Nevertheless, the most fundamental approach to improve human cell engraftment and to overcome still existing innate immunity has been the alteration of strain background and addition of mutations.

Balb/c-*scid bg* and CB57BL/6J-*scid bg* mice both contain the *Prkdc*^{scid} as well as a mutation in lysosome trafficking regulator (*Lyst*^{bg}). This resulted in strains with no B or T cells, neutropenia/granulocyte defects and decreased NK cell activity. The Balb/c-*scid bg* was also radiosensitive [13, 14]. However, leakiness remained a problem in these *scid bg* strains. None-obese diabetic (NOD) mice with *scid* mutation (*Prkdc*^{scid}) showed an altered antigen expression, imperfect myeloid lineage production, lack of complement and low NK cell activity, with better engraftment levels of human cells compared to the *CB17-scid*. This strain has been crossed further to become NOD/LtSz-*scid*, which remained diabetes-free and has often been abbreviated as NOD-*scid* in literature. The NOD-*scid* mice were highly radiosensitive [8, 15, 16]. Although these NOD-*scid* mice are still known as the 'golden standard' for xeno-transplantation studies, leakiness limit their usage and 70% of these mice develop thymic lymphomas, severely decreasing their life span [15, 17]. A knock-out of b2-microglobuline in NOD-*scid* mice (NOD-*scid-B2m*$^{-/-}$) showed an improved engraftment rate compared to the usual NOD-*scid* mice. In these mice homozygosity for the *B2m*$^{-/-}$ allele resulted in the absence of MHC class I expression and thereby loss of NK cell activity [18]. Unfortunately, the NOD-*scid-B2m*$^{-/-}$ mice developed lymphomas even faster compared to previously described strains [18] and mice with *B2m*$^{-/-}$ were prone to develop hemochromatosis [19].

Introduction of a *Rag1* or *Rag2* mutation in NOD-*scid* mice solved the leakiness-problem, but engraftment levels in these *Rag*-mice remained low [5, 20, 21] and problems with development of lymphomas, as described in earlier models with NOD-*scid*, persisted [17, 22]. Mice with a knockout of either *Rag1* or *Rag2* have a very similar phenotype to scid-knockout mice in the immune system (elimination of T and B cells), but they do not have the side effect of radiation sensitivity. Combining a *Rag* mutation on a NOD strain background only (NOD-*Rag1*$^{-/-}$) had an engraftment rate comparable to NOD-*scid* mice with better survival rates [22]. *Rag* mice however were more resistant to radiation than NOD-*scid* [8, 22] and needed additional conditioning to attenuate their innate immunity before injecting human cells [5]. In addition, NOD-*Rag*$^{-/-}$ mice still showed late onset of lymphomas like follicular centre cell and thymic lymphomas [22].

Introduction of mutations in the common cytokine receptor y-chain led to more essential improvements. The interleukin 2 receptor gamma (IL-2Rγ) is responsible for correct signal transmission within the abg-complex of interleukins. A complete null mutation of this *Il2rg* fully eliminates the possibility to signal through the y-chain [23] and impairs NK cell development [24]. The generation of NOD-*scid-Il2rg*$^{-/-}$ mice created a model with high engraftment levels without development of thymic lymphomas [23, 25]. Knock-outs with either a truncated *Il2rg*$^{-/-}$ (NOG-mice) or null mutation of this gene (NSG-mice) are known. NSG mice were more efficiently engrafted than NOG mice which indicates that the presence of the extracellular domain of IL2Rγ-chain in

NOG mice might negatively affect human cell engraftment [26]. The combination of both NOD and *Il2rg* mutation also made the mice less prone to 'leakiness' [27]. Combining a NOD-*Rag1*$^{-/-}$ mouse with *Il2rg*$^{-/-}$ provided a model (NRG-mice) comparable to NOD-*scid*-*Il2rg*$^{-/-}$. However, NRG-mice do not have the same sensitivity to DNA damage as NSG-mice do. This makes it a suitable model in any application that requires high doses of radiation [28]. The H2^{d}-*Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (also referred to as *Rag2*$^{-/-}$*γc*$^{-/-}$ mice) had no T and B cells and no NK cells. These mice showed neither leakiness nor thymic lymphoma development; even though they were not based on the more commonly used CB17-*scid* or NOD-*scid* background [29]. Their main benefit was the possibility to introduce human cells intravenously, while maintaining high engraftment levels. This reduced the amount of human cells that needed to be injected for response considerably, compared to other strains.

Although strains for humanized mouse models were greatly improved in the past, the perfect strain still remains to be found. Recent studies with HLA transgenic mice show promising results to generate new models with high engraftment rates and prolonged survival [30, 31].

Table 1 shows an overview of mutations in the common strains mentioned above, including phenotype, known limitations, reconstitution materials and known usage for either GvHD or allograft studies. A detailed overview of the advantages and disadvantages of most of these strains has also recently been reviewed by Shultz et al [32, 33].

Reconstitution

Material choice

In the development of a humanized mouse model an adequate source should be chosen to create an actual immune system in the immunodeficient animals. So far, a few sources to repopulate these mice are known: (1) fetal cells, (2) human peripheral blood leukocytes, (3) human stem cells, and (4) induced pluripotent stem cells (iPSCs).

The first technique consists of transplanting human fetal liver cells, human fetal thymus and human fetal lymph nodes and was first developed in *scid*-mice. The fetal liver holds all the progenitors for myelomonocytic, erythroid and lymphoid lineages. As the cells are obtained early in fetal stage, the T cells are not yet committed to self and non-self-recognition. This results in donor human T cells which fail to provoke a graft-versus-host (GvH) response. The fetal thymus is necessary to induce central tolerance for immature human T cells. Finally, the addition of fetal human lymph nodes promotes human B cell development. These so called SCID-Hu mice have human CD4^{+} and CD8^{+} T-cells and their circulation contains human IgG. However, usage of this model requires high surgical skills [3].

Table 1. Overview of common humanized mouse strains, phenotype, limitations, known use and reconstitution material.

Mouse common name	Mutation(s)	Phenotype
CB17-*scid*	*Prkdc*^{scid}	• No mature T cell • No mature B cell • Radiosensitive
NOD-*scid*	*Prkdc*^{scid} Lt or LtSz	• No mature T cells • No mature B cells • Radiosensitive • Decreased innate immunity • Decreased NK cell activity • Defective macrophages
NOD-*Rag1*	*Rag1* Lt or LtSz	• No mature T cells • No mature B cells • Radiation resistant
NOD-*Rag2*	*Rag2* *Lt or LtSz*	• No mature T cells • No mature B cells • Radiation resistant
Balb/c-*scid bg*	*Prkdc*^{scid} *Lyst*^{bg}	• No mature T-cells • No mature B cells • Neutropenia • Decreased NK activity • Radiosensitive

Limitations	Known study subjects	Known reconstitution material	Reference
• Low level engraftment of human cells • Anergic human T cells • Xenospecific selection T cell repertoire • Leakiness of T and B cells • Enhanced innate immune system	• Acute GvHD • Allograft studies	• Surgical implantation or intravenous injection of human fetal thymus, liver, lymph node and spleen tissue • Human bone marrow transplant with additional EPO, hu-MGFor PIXY321 • Intraperitoneal injection of huPBL • Human fetal lung tissue transplant	3-7, 46, 76
• Leakiness of T and B cells • Thymic lymphoma development • Better engraftment when injected in bone marrow compared to intravenous injection	• Possible GvHD (not further specified)	• Intra peritoneal injection of huPBL (with or without antiCD122) • Intravenous injection of human T lymphoblastoid cells • Intrafemoral injection of huCD34+ cord blood cells	5, 7, 15, 16,26, 43
• Low and/or variable engraftment of human cells • Late onset lymphoma development • Might need additional conditioning to attenuate innate immunity		• Intra peritoneal injection of huPBL	5,17,22
• Low and/or variable engraftment of human cells (especially after i.p. injection) • Late onset lymphoma development • Might need additional conditioning		• Human thyroid tissue transplant • Intra peritoneal injection of huPBL	20, 21
• Leakiness • Low level engraftment of human cells	• Allograft studies	• Intravenous injection of thymocytes • Human internal mammary artery graft (with or without intra peritoneal huPBL injection)	13-15,52, 66, 67, 69

Table 1. Continued.

Mouse common name	Mutation(s)	Phenotype
NOD-*scid B2m*$^{-/-}$	*Prkdc*^{scid} Lt or LtSz b2-micro-globuline	• No mature T cells • No mature B cells • Lack of MHC class I expression • Low NK cell function • Radiation sensitive
NRG (NOD-*Rag1*$^{-/-}$ *IL2rg*$^{-/-}$)	*Rag1* Lt or LtSz *Il2rg*	• No mature T cells • No mature B cells • Impared NK cell development • Radioresistant
NSG (NOD-*scid IL2rg*$^{-/-}$)	*Prkdc*^{scid} Lt or LtSz *Il2rg (null mutation)*	• No mature T cells • No mature B cells • Impared NK cell development • Radioresistant
NOG (NOD-*scid IL2rg*$^{-/-}$)	*Prkdc*^{scid} Lt or LtSz *Il2rg (truncated)*	• No mature T cells • No mature B cells • Impared NK cell development
(Balb/c or H2^{d}) *Rag2*$^{-/-}$ *yc*$^{-/-}$	*Rag2* *Il2rg*	• No mature T cells • No mature B cells • No NK cells • Radioresistant

	Limitations	Known study subjects	Known reconstitution material	Reference
	• Rapid lymphoma development • haemochromatosis	• Acute GvHD	• intravenous injection of human T (blastoid) cells	18,44, 48
	• Comparable to NOD-*scid IL2rg*$^{-/-}$)	• GvHD (not further specified)	• Intravenous injection of huPBL	28,30
	• Higher engraftment levels of human cells in newborns compared to adults • Xenospecific selection of human T cells might occur • However: high engraftment without irradiation is possible	• Acute GvHD • Sclerotic chronic GvHD	• Intravenous injection of huCD34+ cord blood cells • Intrafemoral injection of huCD34+ cord blood cells • Intravenous injection of huPBL	23, 25,26, 45, 50, 43
	• Less efficient engraftment of human cells compared to NSG mice	• GvHD (not further specified) • Allograft studies	• Intrafemoral injection of huCD34+ cord blood cells • Human liver transplantation	24, 26, 43, 74
	• Additional conditioning might be necessary • Variable human cell engraftment has been described • More constant model for allograft studies	• Sclerotic chronic GvHD • Allograft studies	• Intravenous injection of huCD34+ fetal liver cells (with or without human thymus transplant) • Intravenous injection of huPBL • Human internal mammary artery with intra peritoneal huPBL injection • Human internal mammary artery branch with intravenous human CD25-CD4+ T cells	29, 32, 40, 51, 59, 61, 68

The second technique uses injection of human peripheral blood leukocytes (huPBLs) and is much easier. The level of engraftment appears to be higher compared to the SCID-Hu model [34]. Injection of PBLs ip in *scid*-mice resulted in a transfer of various cell types, including human haematopoietic stem cells (HSCs) and mature T and B cells. These cells are able to generate a functional immune response [6]. Due to the mature portion of immune cells injected, this model provides a fast way to study the human immune system *in vivo* [28].

The third technique includes injecting of human HSCs. Sources for human HSCs are umbilical cord blood (UCB), bone marrow, cytokine-mobilized peripheral blood, or fetal liver [25]. Known examples of this technique are injection of early hematopoietic stem cells (CD34+) of huUCB in the liver of *Rag2*$^{-/-}$*yc*$^{-/-}$ [35], injecting human UCB cells via the facial vein into sub-lethally irradiated NOD-*scid Il2rg*$^{-/-}$ new-born mice [23] and NOD-*scid Il2rg*$^{-/-}$ mice injected with cytokine-mobilized peripheral blood stem cells [25, 36]. In addition, Nakauchi H *et al*. has recently reported that *in vitro* generation of hematopoietic stem cells from induced pluripotent stem cells has the potential to provide a novel approach for developing humanized mice [37].

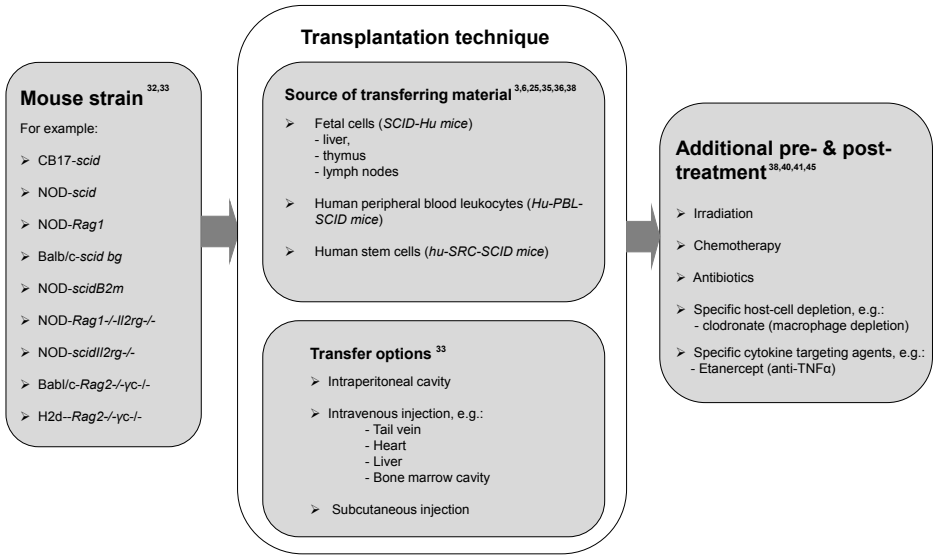

Figure 1. Flowchart for designing a humanized mouse model. This flow charts shows an easy overview of usable murine strains, sources and transfer techniques and possible pre- and post-treatment options that might be used to design a humanized mouse model for either GvHD or allograft rejection research, including references.

Transfer options

The transfer route of the human cells into the mice is a critical determinant for establishing a suitable humanized mouse model [34]. A widely used method is injection of human cells into the peritoneal cavity. Other options are intravenous injection in tail vein, heart or liver. Less commonly, injection in the bone marrow cavity has been applied. When SCID mice were injected in the peritoneal cavity with human PBLs, cells could be detected in that area for minimally 3 weeks. In other organs, cells were detected after one month, predominantly T-cells (96-100%, $CD4^+$ or $CD8^+$), with a peak between one and two months. The amount of cells declined at five months [6]. Mainly spleen and bone marrow, and to a lesser extent lung and liver, were infiltrated, but too many cells in these organs causes (un)desired illness e.g. graft-versus-host disease.

T cells appeared to be an important cell type to create an immune system *in vivo*. However, accessory cells were necessary. When injected solely, T cells could not populate *scid*-mice. This indicates that human accessory cells, like dendritic cells and macrophages, present in the PBL mixture play an important role in immune activation [5]. This was also indicated by injection of PBLs intravenously (i.v.). Apparently, it is harder to get proper bystander help from accessory cells to activate T cells when injecting i.v. [6]. Comparable to the i.v. route via the tail vein in adult animals is the intravenous method via the cardiac route in new-born animals [38]. Intracardiac injection of huHSCs in newborn *Il2rg*$^{-/-}$ mice showed more efficient engraftment than injection via the same route in adult mice [39].

Bearing the importance of accessory cells in mind some other routes of injection have been studied but are less frequently used, like an intrahepatic injection route in conditioned newborn *Rag2*$^{-/-}$*yc*$^{-/-}$ mice with CD34+ human cord blood cells [35] and direct delivery of huHSCs into the bone marrow cavity of the femur [26].

Pre- and post-treatment options

Pre- or post-treatment of the mice might be added. Known basic pre-treatment regiments include radiation (for example total body irradiation), chemotherapy or a mix of both. Other pre-treatment options are targeting the remaining murine cells or introduced donor derived cells, e.g. the depletion of macrophages with clodronate-containing liposomes [40], additional EPO, human mast cell growth factor (hu-MGF), PIXY321 (a fusion protein of IL-3 and GM-CSF) or anti-CD122 mAb for NK cell depletion [4,5]. Nevozhay et al [41] provided an overview of some protocols, including irradiation protocols with known effective doses in different murine models based on currently described protocols [38]. Post-treatment options include e.g. antibiotics, to prevent infections or other medication to prevent GvH reaction.

APPLICATIONS

Graft-versus-host disease

Humanized mouse models can serve as excellent models for Graft-versus-host disease (GvHD) related research, especially when focussing on treatment with e.g. specific cell-targeting medication to reduce the GvHD.

GvHD is a major complication occurring after allogeneic bone marrow transplantation (BMT) or donor lymphocyte infusions in immunodeficient recipients that may lead to severe morbidity or even death. Since the 'perfect model' to study the complete GvH responses remains lacking, knowledge of the exact pathogenesis of GvH responses still relies on picking the right model for the right experiments. General considerations important for selecting a proper model for GvHD research from the current pool of immunodeficient strains have well been outlined earlier by Schoeder *et al* [42]. One of the benefits of an humanized mouse model is the possibility to differentiate between host- or donor related reactions, e.g. by differentiating between species specific cytokines or cells.

Due to different kinetics, the distinction between acute and chronic GvHD (aGvHD and cGvHD respectively) in mice cannot be based on time. Differentiation between acute and chronic GvHD relies on comparison of the symptoms and morphological aspects to the human GvHD equivalent. For chronic GvHD, one can differentiate between a sclerotic and an autoantibody-mediated variant. In mice the differentiation between aGvHD and cGvHD is primarily made on the predominant T cell subset (Th1 in aGvHD and Th2 in cGvHD), specific cytokine production (e.g. TGF-α, IL1 in aGvHD or TGF-β in cGvHD) and/or the presence of for example autoantibody production and/or systemic fibrosis [42].

Humanized mouse models known to be used in GvH research are NOG [43], NOD-*scid* [43], NOD-*scid-B2m*$^{-/-}$ [44], NOD-*scid Il2rg*$^{-/-}$ [45] and the *Rag2*$^{-/-}$*yc*$^{-/-}$ mice [40]. Although many of the earlier discussed humanized mouse models have been presented with an analysis of the specific cell subtype in the human infiltrate, less effort was often made to differentiate them as a specific model for aGvHD or sclerotic versus autoantibody-mediated cGvHD.

For aGvHD the CB17-scid with i.p. huPBL injection has been suggested. In this model the similarity to the huGvHD equivalent was based on the production of macrophages migration inhibitory factor (MIF) [46], although the suggestion of MIF to relate to acute huGvHD has been

made on a mouse-mouse based model [47]. NOD-*scid Il2rg*$^{-/-}$ was suggested as a model for aGvHD as well, based on the presented delaying effects of etanercept (an anti-TNFα agent) on the onset of the GvHD symptoms in this model [45]. Intravenous injection is possible in these mice, in which a small number of huPBL's is efficient to induce GvHD and total body irradiation was not always necessary [43]. Furthermore, the NOD-*scid-B2m*$^{-/-}$ model injected with human T cells could be considered a model for aGvHD and is one of the few models with a specified scoring system for the occurring acute reaction [44, 48].

The autoantibody-mediated variant of chronic GvHD is not common in humanized mouse models, since humanized mouse models usually lack the ability to produce (autoreactive and other) T cells due to the lack of thymic tissue. More often, humanized mouse models with autoantibody production have been used to study other autoimmune diseases like SLE [49]. *Rag2*$^{-/-}$*yc*$^{-/-}$ injected with huPBL from a donor suffering from SLE or huPBL injection in CB17-*scid* or CB57BL/6J-*Il2rg*$^{-/-}$ mice after human thyroid engraftment [21] have been used to study human auto-immune thyroiditis, both containing auto-reactive antibodies from the donor.

For sclerotic cGvHD the NOD-*scid Il2rg*$^{-/-}$ and the *Rag2*$^{-/-}$*yc*$^{-/-}$ have been used previously [40, 50]. The morphologic changes are best known for the *Rag2*$^{-/-}$*yc*$^{-/-}$ mice [51].

In general, in a humanized model for GvHD research the choice of material-transfer will influence the developing GvH response; injection of huPBLs is said to represent a more acute GvHD model (even though sclerotic cGvHD could be achieved using appropriate conditions), whereas the transplantation of bone marrow, thymus and liver represents a more chronic model [50, 51]. The rate and severity of the reaction will depend on several items. Mice injected with HSCs depleted of human T cells showed less GvHD development [38]. Also, SCID and NOD-*scid* mice usually showed low engraftment, whereas models incorporating other mutations, sometimes combined with extra preconditioning, lead to higher engraftment levels of human cells and therefore more GvH response [40, 45]. NOD-*scid Il2rg*$^{-/-}$ mice showed high levels of engraftment even with low amounts of huPBL injection i.v. and *Rag2*$^{-/-}$*γc*$^{-/-}$mice injected intravenously with huPBL also produced a stable model with development of systemic GvHD symptoms. In virtually all mice the morphology and rate of the GvHD symptoms could be influenced with macrophage-depletion as preconditioning. Macrophage-depleted mice developed severe, more acute symptoms and non-macrophages-depleted mice showed a slower development of a more sclerotic GvH response [40].

Keep in mind that although high engraftment levels in your model comes with more intense clinical GvHD symptoms [5], the symptoms might not correlate with the human GvHD phenotype. It is only proof that the injection of human cells in an immunodeficient mouse induces a GvH reaction. With the difficulties in translation of the induced GvH reaction in a comparable acute and chronic equivalent of human GvHD, the use of histology, cytokine analysis and infiltration analysis remains essential in every GvHD study using a humanized model.

Host-versus-graft disease (allograft rejection)

Solid organ transplantation often suffers rejection of the donor organ by the host, known as a 'host-versus-graft' response (HvG) or allograft rejection. Despite immunosuppressive medication, rejection remains a problem that restricts long term success of graft survival [52, 53]. One aim of immunologists in transplantation research is realizing graft survival without chronic immunosuppression [54, 55]. To achieve this goal humanized mouse models can be used in experimental studies, to analyse the underlying mechanisms of rejection and to evaluate several reagents, because of closer resemblance to the human immune system than other models. For example in allograft vasculopathy; mouse-mouse/rat-rat transplantation models provided a different mechanism with influx of smooth muscle cells [56-58] compared to the humanized mouse models which showed influx of mononuclear cells [59-61]. Non-humanized models give us insight in the process, but do not mimic the human immune response as humanized mouse models do.

The immunodeficient mice described before, tolerated allogeneic grafts (from a different mouse of the same species) and even xenogeneic grafts (different species) [3] and allowed engraftment of human immune cells. Together, these properties made them suitable models for HvG rejection research. A recent review by Brehm and Shultz described the historical perspective of humanized mice in allograft rejection studies [62]. They discussed skin and islet allografts in detail [63-65] and to a lesser extent other allografts. This paragraph will focus on rejection studies of solid organ transplantation, but also vessel rejection studies shall be included.

One of the first 'humanized' mouse models used for transplantation studies was the SCID/bg (Balb/c-*scid bg*) mouse, with pig epicardial coronary arteries inserted into the murine infrarenal aorta [66]. Also human arteries have been successfully transplanted [67]. When mice transplanted with human arteries were also engrafted with huPBLs from an allogeneic donor an immune response against the arterial graft occurs *in vivo*. This model made it

possible to study aspects of post-transplant graft arteriosclerosis with close resemblance to human graft arteriosclerosis [52]. When comparing the *Rag2*$^{-/-}$*yc*$^{-/-}$ model to the well-established SCID/bg model for vascular allograft rejection, *Rag2*$^{-/-}$*yc*$^{-/-}$ mice were advantageous on both engraftment of human artery as well as engraftment of huPBLs. These mice did not become leaky, as they do not involve the SCID mutation. *Rag2*$^{-/-}$*yc*$^{-/-}$ mice provided a more constant model, with only some discrete intima changes preceding the huPBL injection compared to SCID/bg [59, 61, 68]. However, a limitation of the hu-PBL-SCID model is that from all injected human cells only T cells and a few B cells survive.

For studying macrophages in allograft rejection a new model was introduced. HSCs were isolated from adult human peripheral blood enriched for CD34^{+}. Two strains (NOD-*scid Il2rg*$^{-/-}$ and SCID/bg mice) have been compared for their ability to support human skin, artery and HSC engraftment. SCID/bg animals were advantageous over NOD-*scid Il2rg*$^{-/-}$ for the engraftment of human skin and artery, but engraftment levels of HSCs were higher for NOD-*scid Il2rg*$^{-/-}$. This was disappointing, because a proper working model needs all engraftments on a relative high level to show human macrophages inside skin or artery. Creating a model in which both macrophages and T cells can be transferred, lead to artery engraftment in SCID/bg mice with only HSC injection. The intima of the graft in SCID/bg animals showed proliferation and presence of macrophages, enabling it as a model to study the role of macrophages [69].

Looking at the history of humanized mouse models used in allograft studies, one will notice that most models used for transplantation studies were developed in the field of microbiology and cancer research. Instead of transplanting a human artery one might consider transplanting parts of solid organs. However, this is a whole new field of organ rejection studies since complete solid organs will not fit the size of a mouse. An example of humanized mouse model usage in microbiology research using solid organ tissue is the model constructed for liver research. In this model mouse hepatocytes were replaced by human hepatocytes [70]. This model was used to study hepatitis B [71] and hepatitis C virus [72]. Functional analysis of human liver was assessed within these models in Ncr-Nude mice [73] and NOG mice [74]. Another example is the model from the Crombleholme group in which a human fetal trachea was transplanted on the flank of SCID or SCID/bg mice [75-78]. They showed development of human airway epithelium and submucosal gland [79]. This model is now used to study gene therapy for cystic fibrosis. Rejection in lung transplantation is now studied with mouse models in which a trachea is transplanted from one mouse to another [80]. These models for lung and liver transplantation might eventually be used for transplant rejection studies as well.

DISCUSSION

In the past few decades, a lot of progress has been made on the development of humanized mouse models to study the human immune system in transplantation [33, 62].

Despite the major improvements so far to enable both GvHD and allograft rejection studies, lots of improvement maintains necessary, before all transplantation processes and different organs of interest for transplantation (like e.g. human brain, lung, and gut) can be studied within such a model[81].

The pathogenesis of GvHD remains very complex and new insights in the effects of many different cell-subsets and their relations in the development of GvHD are still uncovered. An important issue that still needs to be addressed in humanized mouse models for GvHD is the lack of autoreactive antibody production by the recipient. Also, extra effort will have to be made to ensure stability of engraftment levels and clinical symptoms in the models. More insight in the morphological comparisons and differences of the humanized models to acute and chronic (sclerotic or autoantibody-induced) huGvHD needs to be obtained. This includes improvement of our knowledge of migration patterns of donor cells within the murine organs with histopathological studies. We quite often assume that the morphological aspects of the reaction are in line with the severity of the clinical aspects, but when studying huGvHD, proper evaluations of histological severity of the reaction versus actual clinical outcome are quite rare. For evaluation of this aspect in a murine model, grading systems for the morphology of the induced GvH reactions in humanized models are currently scarce.

Within the research field of allograft rejection there are size limitations to study human organs. One cannot transplant a human kidney or lung within a mouse and is therefore limited to little pieces or parts of the organ. Currently, parts of the rejection process (e.g. artery or skin) can be studied and although these are well established models, they are still just little components of the complete rejection pathogenesis. In artery transplantation and huPBL infusion a fierce lymphocytic rejection process is mimicked, but the role of the humoral immune system or chronic fibrotic processes are excluded.

With the newest immune deficient mice, new techniques for engraftment of the human immune system and improvements on transplantation techniques better models will be created. For both clinical transplantation research and GvHD research this is very important, since well-established models will improve our knowledge about the pathogenesis and new

therapeutics can be tested. For this reason, it is important that the field of basic science collaborates with clinical scientists and that together they try to overcome rejection difficulties.

When choosing a model for your research, you should strongly focus on the hypothesis you want to test. In this review we outline known phenotypes, limitations and known usage for common strains of humanized mouse models in GvHD and allograft studies (summarized in Table 1) and the methods to create these models with regard to reconstitution material and transplantation methods (summarizing the steps to take in Figure 1), that will help researchers to choose the right model for their research in either GvHD or allograft studies. From the very beginning you should look for a model that mimics the process you would like to study as close as virtually possible with regards to the current limitations that remain in humanized models. One should realize that using a humanized mouse model mimics the human situation as closely as possible, but still does not include all facets of the human system and is based on a combination of two different species. However, when the right model is chosen, you are just one step away to contribute to the improvement on current knowledge about allograft rejection and graft-versus-host rejection.

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CHAPTER 3

HISTOLOGICAL ASSESSMENT OF THE SCLEROTIC GRAFT-VERSUS-HOST RESPONSE IN THE HUMANIZED RAG2-/-γC-/- MOUSE MODEL

Marieke C.H. Hogenes[1], Suzanne van Dorp[2], Joyce van Kuik[1], Filipa R.P. Monteiro[1], Natalie ter Hoeve[1], Marijke R. van Dijk[1], Anton C. Martens[3,4], Roel A. de Weger[1].

[1]Department of Pathology,
[2]Department of Hematology (DIGD),
[3]Department of Cell Biology,
[4]Department of Immunology.

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ABSTRACT

Graft-versus-host disease (GvHD) still remains a frequently occurring and difficult to treat complication in human allogeneic stem cell transplantation. Murine transplantation models are often used to study and understand the complex pathogenesis of GvHD and to explore new treatment-strategies. Although GvHD-kinetics in murine models may differ from the human counterpart, for identification of the crucial factors responsible for the major pathology in GvHD adequate models are essential.

In this report we present a detailed description of the specific histological features of a graft-versus-host induced fibrotic response in xenogeneic RAG2$^{-/-}$γc$^{-/-}$ mice, after total body irradiation and injection with human peripheral blood mononuclear cells (huPBMC's). We describe the full morphological feature of this reaction, including a detailed analysis of the specific tissue infiltration patterns of the huPBMC's. Our data show the development of fibrosis, predominantly near blood vessels, and reveals different cell-populations and specific cell-migration patterns in the affected organs. The combination of immunohistochemical cell characterization and mRNA expression analysis of both human (donor) and murine (host) derived cytokines reveal an interaction between host tissues and donor derived cells in an entangled cytokine profile, in which both donor- and host derived cytokines contribute to the formation of fibrosis.

INTRODUCTION

Graft-versus-host disease (GvHD) is a major problem in allogeneic stem cell transplantation, causing late morbidity and mortality [1], affecting a wide range of organs [2]. One of the difficulties in combating GvHD is a lack of understanding of the pathophysiology of the syndrome [3]. Steroid therapy and calcineurin inhibitors are currently the most common pharmacologic agents used for treatment and/or prevention of GvHD, although other cells, like B cells, dendritic cells, macrophages and regulatory T cells (Tregs) might also be relevant therapeutic targets [4-10].

To study human GvHD development *in vivo*, several different experimental models are used, based on immunodeficient mouse strains, including severe combined immunodeficient (SCID) mice, non-obese diabetic severe immunodeficient (NOD-SCID) mice and the RAG2$^{-/-}$γc$^{-/-}$ mice [11-15]. Despite the fact that several xenogeneic mouse models are reported [15-20] , only a few of these are referred to as specific GvHD models [15, 21-23]. In these mice, human peripheral blood mononuclear cells (including lymphocytes) are injected into the mice and the subsequently developing GvHD is studied. To assess the development of this graft-versus-host (GvH) reaction, most studies focus on the clinical appearances and associated parameters of the mice. However, little is known of the histopathological origin that forms the basis of these "clinical" aspects whereas the histological patterns and topographic location of the different human and murine inflammatory cells receive minimal attention [15, 21, 23-25].

In this paper, we present a detailed (immuno)histochemical analysis of the localization of human immune cells in various organs as well as the sites of GvH-induced fibrosis in the RAG2$^{-/-}$γc$^{-/-}$ mouse model. In an attempt to identify the trigger of fibrosis, we used quantitative PCR to measure the mRNA expression of human and murine cytokines in affected tissues. This will allow us to answer the question whether fibrosis is induced by human cytokines directly or by murine cytokines in a reaction to the damage inflicted by human cells.

MATERIALS AND METHODS

Mice and conditioning regiments

RAG2$^{-/-}$γc$^{-/-}$ and Balb/c mice were obtained from the Netherlands Cancer Institute (Amsterdam, The Netherlands) [26]. The mice were bred and maintained in filtertop cages under specified pathogen-free conditions at the Central Animal Laboratory Institute (Utrecht University, The Netherlands) and were given sterilized food pellets and sterile water *ad libitum*. The experiment was performed twice.

In each experiment 10 female mice at 15-16 weeks of age received total body irradiation with a single dose of 350 cGy (gamma irradiation from a linear accelerator) and were injected intravenously (iv) with human peripheral blood mononuclear cells (huPBMC's) on the following day. Control groups of 5 RAG2$^{-/-}$γc$^{-/-}$and 2 Balb/c mice were irradiated, but did not receive huPBMC's. Tissues from both control groups were used, to test for specificity of human reagents.

Mice were weighed once a week and sacrificed by cervical dislocation when losing more than 15% of their original body weight or when having severe GvH associated morbidity. The survival varied between mice, from day 29 up to day 70.

Experiments were conducted after acquiring permission of the local Ethical Committee for Animal Experiments and in accordance with the Dutch law on animal experimentation.

Preparation and transplantation of huPBMC's

HuPBMC's were prepared as described by van Rijn et al [15]. Briefly, fresh buffy coats were obtained from healthy human volunteers at the Bloodbank of the University Medical Centre Utrecht and huPBMC's were isolated using Fiqoll Hypaque (Pharmacia, Uppsala Sweden) density centrifugation. Cells were washed twice in phosphate-buffered saline (PBS) and resuspended in PBS/0.1% human serum albumin (HSA). Fresh cell suspensions of 0.2 ml, containing 15-30 x 10^6 huPBMC's were injected iv in the tail vein, without prior ex-vivo stimulation, using a different human donor in each experiment.

Histology and immunohistochemistry

From each mouse, the spleen, lungs, liver, ileum and colon, kidneys, skin, heart and femur, were isolated for morphological analysis, adding up to a total of n=20 per organ (in 2 experiments with 10 mice each).

Each organ was split; one part was frozen and stored in liquid nitrogen and another part was fixed in PBS-buffered formaldehyde (4%). The femur was decalcified with EDTA, to enable accurate bone marrow analysis. The formalin fixed organs were embedded in paraffin and tissue sections were cut at 4 µm using coated slides, for staining with haematoxylin-eosin (H&E) for histology and several primary antibodies for immunohistochemistry (Table 1).

Immunohistochemical staining was performed in a Bond-max™ automated immunostainer (Leica Microsystems, United Kingdom). Slides were pre-treated automatically with either citrate, using the Bond Epitope Retrieval solution 1 (AR9961), or EDTA, using the Bond Epitope Retrieval solution

2 (AR9640). Washing steps between each reagent were performed using 1x Bond Wash Solution. The staining was identified by a polymer based detection system with Diamino-benzidine (DAB) using the Bond Polymer Refine Detection kit (DS9800). This detection includes incubation with Post Primary for 15 min, Polymer for 8 min and DAB for 10 min. The sections were counterstained with haematoxylin.

For human FoxP3 staining slides were deparaffinized, washed and blocked with endogenous peroxidase blocker. Antigen retrieval was performed using citrate pre-treatment; slides were then washed and incubated with diluted anti-human FoxP3 antibody for 1 hr. After rinsing, slides were incubated for 30 min with rabbit-anti-rat-HRP (DAKO, Glostrup, Denmark), diluted at 1:250, and incubated with Powervision Goat-anti-rabbit IgG HRP (Immunologic, Klinipath, the Netherlands), followed by DAB staining for 10 min after washing with PBS. Slides were counterstained with haematoxylin.

Mouse-CD68 staining was performed on frozen tissue sections cut at 8 µm. Slides were fixed in acetone for 10 min, air dried, rinsed in PBS and incubated with primary antibody for 1 hr. After rinsing, the slides were fixed in formaldehyde and blocked with endogenous peroxidase blocker, before incubating with a combination of rabbit-anti-mouse antibody-HRP (DAKO) and 10% mouse serum (DAKO) in a dilution of 1:100. Staining was identified using DAB and counterstaining with haematoxylin.

Cross-reactivity of all monoclonal and polyclonal antibodies used (against human antigens), was tested using both a positive control (human tonsil) and a double negative control in every run. The latter consisted of a control staining using BALB/c tissue and murine tissue from RAG2$^{-/-}$γc$^{-/-}$ mice without huPBMC's. In these RAG2$^{-/-}$γc$^{-/-}$ mice without huPBMC's, a control was run with primary antibody and a control staining using murine tissue with human cells without the primary antibody to rule out both false positive staining due to cross-reactivity of the other reagents used, and false negative staining for the primary antibody. Cross-reactivity of anti-murine CD68 was tested in human tonsil tissue.

Slides were assessed for presence, severity and location of both fibrosis and cell infiltrate, including the huPBMC's and murine macrophages by two experienced pathologists using light microscopy. Severity of infiltrate, with respect to B cells, T-helper cells, cytotoxic T cells and plasma cells was scored in a scale of 0-4, as no infiltration (score 0), sporadic / less than 5% infiltration (score 1), mild infiltration varying between 5-25% (score 2), moderate infiltration of 25-50% (score 3) or severe infiltration of more than 60% (score 4). Since the presence of Tregs in human blood varies from 1-10%, different thresholds had

Table 1. Antibodies used for immunohistochemical detection of human cells injected in RAG2$^{-/-}$γc$^{-/-}$ mice.

Primary Antibody	Manufacturer	Animal	Monoclonal or Polyclonal
Hu-CD2	Novocastra	Mouse	Monoclonal
Hu-CD4	Monosan Xtra	Mouse	Monoclonal
Hu-CD8	DakoCytomation	Mouse	Monoclonal
Hu-CD20	DakoCytomation	Mouse	Monoclonal
Hu-CD79a	DakoCytomation	Mouse	Monoclonal
Hu-CD138	Serotec	Mouse	Monoclonal
Hu-κ	DakoCytomation	Rabbit	Polyclonal
Hu-λ	DakoCytomation	Rabbit	Polyclonal
Hu-plasmacell	DakoCytomation	Mouse	Monoclonal
Hu-CD68	Novocastra	Mouse	Monoclonal
Hu-FoxP3	Ebioscience	Rat	Monoclonal
Mouse-CD68	Hycult Biotech	Rat	Monoclonal

to be used to score the presence of human Tregs: no infiltration (score 0), sporadic infiltration of about 1% (score 1), mild infiltration of about 3-5% (score 2), moderate infiltration of more than 5-7% (score 3), severe infiltration of more than about 7% (score 4). Since the percentage of infiltration had to be evaluated by light microscopy, the scores are somewhat objective. A Krippendorff's kappa score of 0.6 was reached when the two pathologists scored individually, but full agreement was achieved by adding the possibility to score intermediate between two consecutive scores when doubt persisted and when scores were re-evaluated by both pathologists together.

Cytokine profile analysis

The cytokine gene expression profile was analysed by quantitative polymerase chain reaction (Q-PCR) to measure the amount of human and murine messenger RNA (mRNA) in the murine tissues. Tissue samples were used from mice injected with huPBMC's and both RAG2$^{-/-}$γc$^{-/-}$and Balb/c control mice without huPBMC's.

RNA was isolated from murine spleen, liver, lung, skin, colon and ileum using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions.

Of each sample 3 µg RNA was incubated with 1 µl of both oligoDT (Promega, Leiden, The Netherlands) and Random Primers (Promega) for 5 min at 70˚C. Finally, cDNA was synthesized by adding a mixture of First strand buffer 5x (Invitrogen, Breda, the Netherlands), 0,1 M DTT, 10mM dNTP's (Invitrogen) and incubation for 1 hr at 42˚C. The cDNA was stored upon use at -20˚C [27].

Clone	Lot	Dilution	Pretreatment
AB75	127119	1:160	EDTA
4B12	247919	1:200	EDTA
C8/144B	51182	1:200	Citrate
L26	83	1:400	Citrate
JCB117	42791	1:200	Citrate
B-B4	605	1:1000	Citrate
-	44399	1:10000	Citrate
-	120	1:20000	Citrate
VS38C	38925	1:400	EDTA
KP1	211708	1:800	Citrate
PCH101	E021753	1:200	Citrate
FA-11	4817M21	1:50	None

For all cytokines and other gene-targets tested, the human and mouse specificity was determined by testing the primer-probe combination against mRNA isolated from normal human and murine tissues (RAG2$^{-/-}$γc$^{-/-}$ and BALB/c mice). Only species specific pre-developed *Taq*Man Assays were used (*Taq*Man Gene Expression Assay: Applied Biosystems, Foster City, CA). The Q-PCR reactions were run on a LightCycler 480 real-time PCR system (Roche Diagnostics, Lewes U.K.). Each sample was run in duplicate and a negative control was used to exclude contamination. GAPDH was used as reference gene. The relative mRNA quantity was determined using the E-method of the LightCycler 480 software (Roche). In this method a correction for PCR efficiency is embedded in the calculation of the relative quantity of gene expression (RQ value)

RESULTS

Clinical symptoms

Mice injected with human cells, all show development of GvHD symptoms (100%), including severe weight loss, ruffled fur, reduced mobility, erythema of the skin and in some cases splenomegaly similar as described previously in other xenogeneic mouse models [15, 23, 28]. Human chimerism levels in peripheral blood varied from 43-100% (first experiment) to 5-51% (second experiment) at time of death, with consistent weight loss in all mice. The pattern of body weight loss was similar as described by R. van Rijn *et al* [15].

Table 2. Statistical analysis of the immunohistochemical data.

			Fibrosis		T Cells	
	N	Survival, Days, Mean (SD)	Histopathology Score, Mean (SD)	P value	Histopathology Score, Mean (SD)	P value
Spleen						
Exp. 1	6	36.7 (7.2)	0.7 (0.8)	.1028	3.2 (0.4)	.0034
Exp. 2	9	39.5 (14.3)	1.8 (1.2)	.0145*	3.7 (0.5)	.0017
Exp.1 vs. exp 2.				.0875		.0769
Liver						
Exp. 1	6	36.7 (7.2)	1.5 (0.8)	.0140*	2.7 (0.5)	.0043*
Exp. 2	9	39.5 (14.3)	1.7 (0.7)	.0021*	2.0 (1.2)	.0137*
Exp.1 vs. exp 2.				.9479		.3268
Lung						
Exp. 1	6	36.7 (7.2)	0.5 (0.5)	.0990	2.7 (0.5)	.0043*
Exp. 2	9	39.5 (14.3)	2.1 (1.0)	.0024*	2.7 (0.5)	.0017*
Exp.1 vs. exp 2.				.0069*		.9429
Skin						
Exp. 1	6	36.7 (7.2)	3.0 (0.9)	.0052*	2.7 (1.0)	.0050*
Exp. 2	9	39.5 (14.3)	1.8 (0.8)	.0023*	2.2 (1.0)	.0024*
Exp.1 vs. exp 2.				.0322*		.04587
Colon						
Exp. 1	6	36.7 (7.2)	0.7 (0.8)	.1028	2.3 (0.5)	.0043*
Exp. 2	9	39.5 (14.3)	2.4 (0.5)	.00019	2.7 (1.2)	.0060*
Exp.1 vs. exp 2.				.00030*		.2800

Mean survival with standard deviation (SD), means scores with SD and p values for histological and immunohistochemical scoring in the most important evaluated organs in experiment 1 (comparing mice injected with huPBMC's to control mice without huPBMC's), experiment 2 (comparing mice injected with huPBMC's to control mice without huPBMC's) and comparing experiment 1 and 2 for differences between the groups of mice with huPBMC's. An example of individual scoring in 4 organs from experiment 2 can be found in table S1 (supplementary data).

B Cells		Plasma Cells		Tregs	
Histopathology Score, Mean (SD)	P value	Histopathology Score, Mean (SD)	P value	Histopathology Score, Mean (SD)	P value
2.0 (0.9)	.0052*	2.3 (1.5)	.0049*	1.2 (0.4)	.0034*
2.4 (1.0)	.0047*	2.1 (1.2)	.0061*	1.2 (1.3)	.0615
	.2716		.9038		.9017
1.2 (0.4)	.0034*	1.5 (1.0)	.0163*	0.3 (0.8)	.4652
0.8 (0.7)	.0277*	0.6 (0.5)	.0548	0.6 (0.5)	.0545
	.2300		.0658		.3074
1.2 (0.4)	.0034*	1.3 (0.5)	.0043*	0.5 (0.8)	.2230
0.8 (0.8)	.0603	0.7 (0.9)	.1114	0.8 (0.4)	.0090*
	.2993		.1171		.2880
0.0 (0.0)	†	1.0 (1.2)	.01041	1.0 (1.2)	.1041
0.0 (0.0)	†	0.0 (0.0)	‡	0.4 (0.7)	.1931
			.1041		.4224
0.0 (0.0)	†	0.0 (0.0)	‡	0.2 (0.4)	.4652
0.0 (0.0)	.5500	0.0 (0.0)	‡	0.1 (0.3)	.5716
	.4960		‡		.7768

* P value is significant

† No B cells detected; t test not possible.

‡ No plasma cells detected; t test not possible

Histological analysis

After injection of huPBMC's many organs, including spleen, lungs, liver, skin, bone marrow, gut and kidneys, were infiltrated with human cells. Since the sacrifice of the mice was based on clinical criteria, the survival of mice varied from 29-70 days. The mice showed development of different degrees of fibrosis in the various organs; the longer mice survived, the more severe the fibrosis in their organs, when compared to the same organs in mice with a shorter lifespan. Mice with higher human chimerism showed more prominent morphological changes as described below, though the pattern of fibrosis and infiltrate migration in both experiments was almost equal and showed clear changes compared to control mice, either as a significant difference or as a trend. In table 2, the number of completely examined mice per experiment, mean survival with standard deviation, mean scores per cell-type per organ with standard deviation and p values are presented. A full description of individual scores of 4 organs in experiment 2 is presented in the *supplementary data (Table S1)*. Out of 20 mice, a total of 15 mice (6 from experiment 1 and 9 from experiment 2) could be fully examined. Five mice died from the GvHD and were lost for evaluation, due to severe post-mortem lytic changes.

Spleen. The spleen contained large amounts of collagenous fibrosis around smaller blood vessels (figure 1). With longer survival time, the severity of the infiltrate increased concurrently and the distribution of the infiltrate became more diffuse. With more extended infiltration through the tissue, the fibrosis spread in the rest of the parenchyma as well, destroying the entire morphology of the spleen.

Lungs and liver. Distribution of fibrosis around blood vessels was also observed in the lung. The fibrosis in the lung concentrated around peri-bronchial blood vessels at all times and to a lesser degree around bronchi and bronchioles when a more extensive infiltrate was found (figure 2). In the liver there was peri-portal fibrosis (figure 3), lined with plasma cells along the lamina limitans, with some bile duct proliferation.

Skin. There was particular extensive fibrosis in the skin (figure 3). The morphology is quite similar to the human sclerodermatous GvH reaction, with a mild lichenoid infiltrate along the epidermal junction, thinning of the epidermal layer, extension of coarse collagen fibres in the dermis at the cost of the underlying subcutaneous fat and loss of hair follicles. Only a few infiltrating human cells were present.

Bone marrow. The bone marrow showed clear signs of fibrosis as well, but the cellularity of the marrow was maintained.

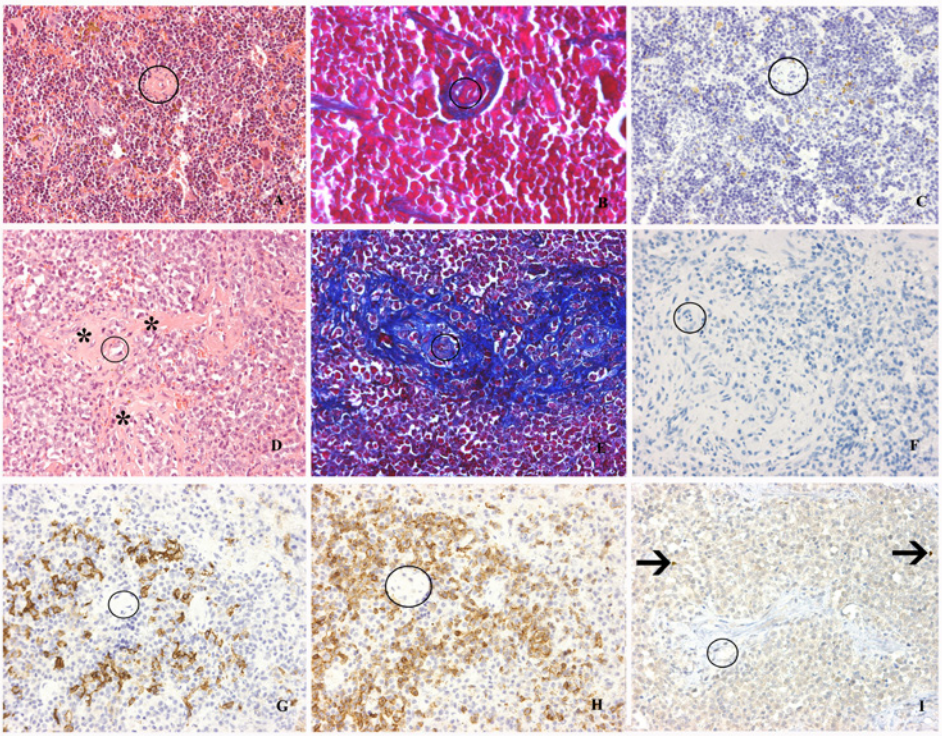


Figure 1. Human infiltrating cells in spleen of RAG2$^{-/-}$γc$^{-/-}$ mice injected with huPBMC.
Infiltrate analysis spleen comparing both histological and immunohistochemical stainings of a control mouse and a mouse surviving 45 days after huPBMC-injection. The displayed morphology is representative for the observed reaction in a total of 15 examined mice (6 mice in experiment 1 and 9 mice in experiment 2).
A. Normal murine spleen, without human cell infiltrate or fibrosis (H&E staining, 20x). B. Normal murine spleen, without human cell infiltrate or fibrosis (AZAN staining, 20x, fibrosis score 0). C. Normal murine spleen, showing no immunohistochemical positivity and no cross-reactivity when staining for human CD2 positive T cells (huCD2 staining, 20x, T cell score 0). D. Perivascular fibrosis in spleen, in a mouse 45 days after huPBMC-injection (H&E staining, 20x, fibrosis score 2). E. Perivascular fibrosis spleen (identical area to D) showing blue staining of the formed collagenous fibrosis (AZAN staining, 20x, fibrosis score 2). F. HuCD68 showing absence of human macrophages in a mouse injected with huPBMC's (huCD68 staining, 20x, macrophage score 0). G. Follicular/perivascular concentration of human B cells in the spleen, after injection with human PBMC's (huCD20 staining, 20x, B cell score 2). H. Diffuse spreading of human T cells in the spleen, after injection of human PBMC's (huCD2 staining, 20x, T cell score 4). I. Focal localization of human Tregs with nuclear positive staining for huFoxP3 after injection with human PBMC's (huFoxP3 staining, 20x, Treg score 1).
Circle: localization capillary blood vessel, asterisk: fibrosis area, arrow: FoxP3 positive Tregs.

Other organs. There were no obvious signs of tissue damage in the gut, in contrast to the human counterpart. The kidneys and heart did not contain signs of tissue damage either, despite the presence of human infiltrating cells (see below for specification).

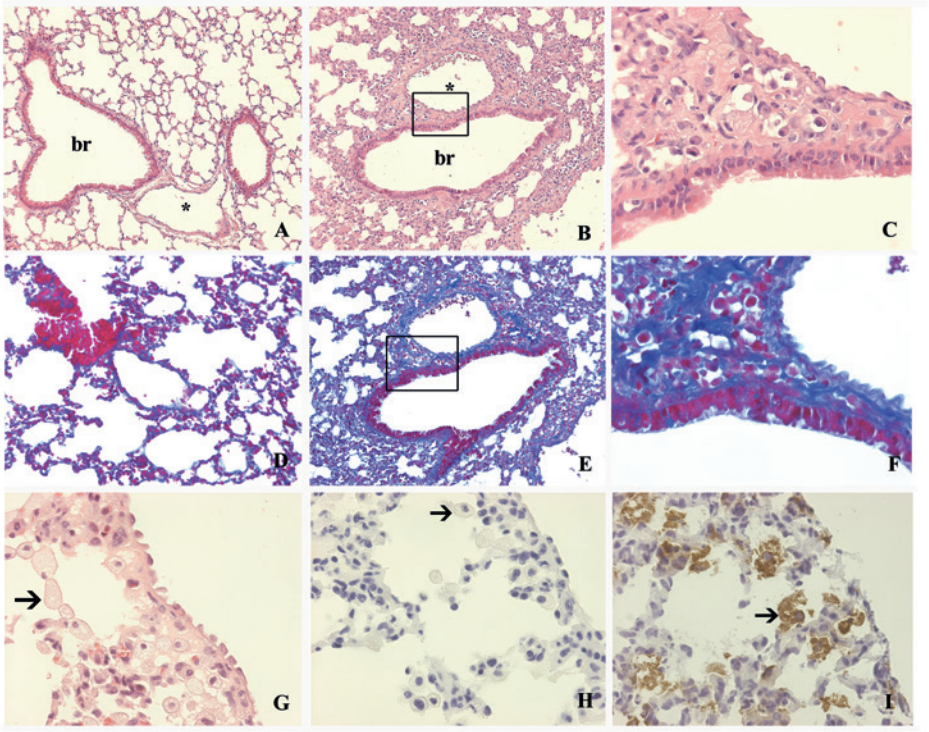


Figure 2. Histological and immunohistochemical analysis of infiltrating human cells in RAG2$^{-/-}$γc$^{-/-}$ lungs. Infiltrate analysis of the lungs comparing a control mouse and a mouse from experiment 1, surviving 45 days after huPBMC-injection. The displayed morphology is representative for the observed pattern in a total of 15 examined mice (6 mice in experiment 1 and 9 mice in experiment 2).

A. Normal murine lung showing thin alveolar septae around bronchi and blood vessels (H&E staining, 10x, fibrosis score 0). B. Lung after injection with human PBMC's, showing peri-vascular and peri-bronchial fibrosis and cell infiltration with hypercellular alveolar septae 45 days after huPBMC-injection (H&E staining, 10x, fibrosis score 1). C. Detail from picture B (inset) showing plasma cells in the area of fibrosis (H&E staining, 40x, plasma cell score 1). D. Normal lung showing thin alveolar septae, without extensive fibrosis around bronchi or vessels (AZAN staining 10x, fibrosis score 0). E and F. showing the same areas of B and C respectively in AZAN staining, with blue staining of the collagenous fibrosis (E. AZAN 10x and F. detail from E (inset) AZAN 40x, fibrosis score 1) G. Foamy alveolar macrophages after injection with human PBMC's (H&E staining, 40x). H. The macrophages do not show human CD68 positivity (huCD68, 40x, identical area to H). I. Positive staining of the macrophages for mouse CD68 confirms murine origin (mouse-CD68, 40x, identical area to H).

Br: bronchus, asterisk: peri-bronchial artery, square: detail area shown in picture C (corresponding to square in B) and F (corresponding to square in D), arrow: foamy alveolar macrophages.

Morphological infiltrate analysis

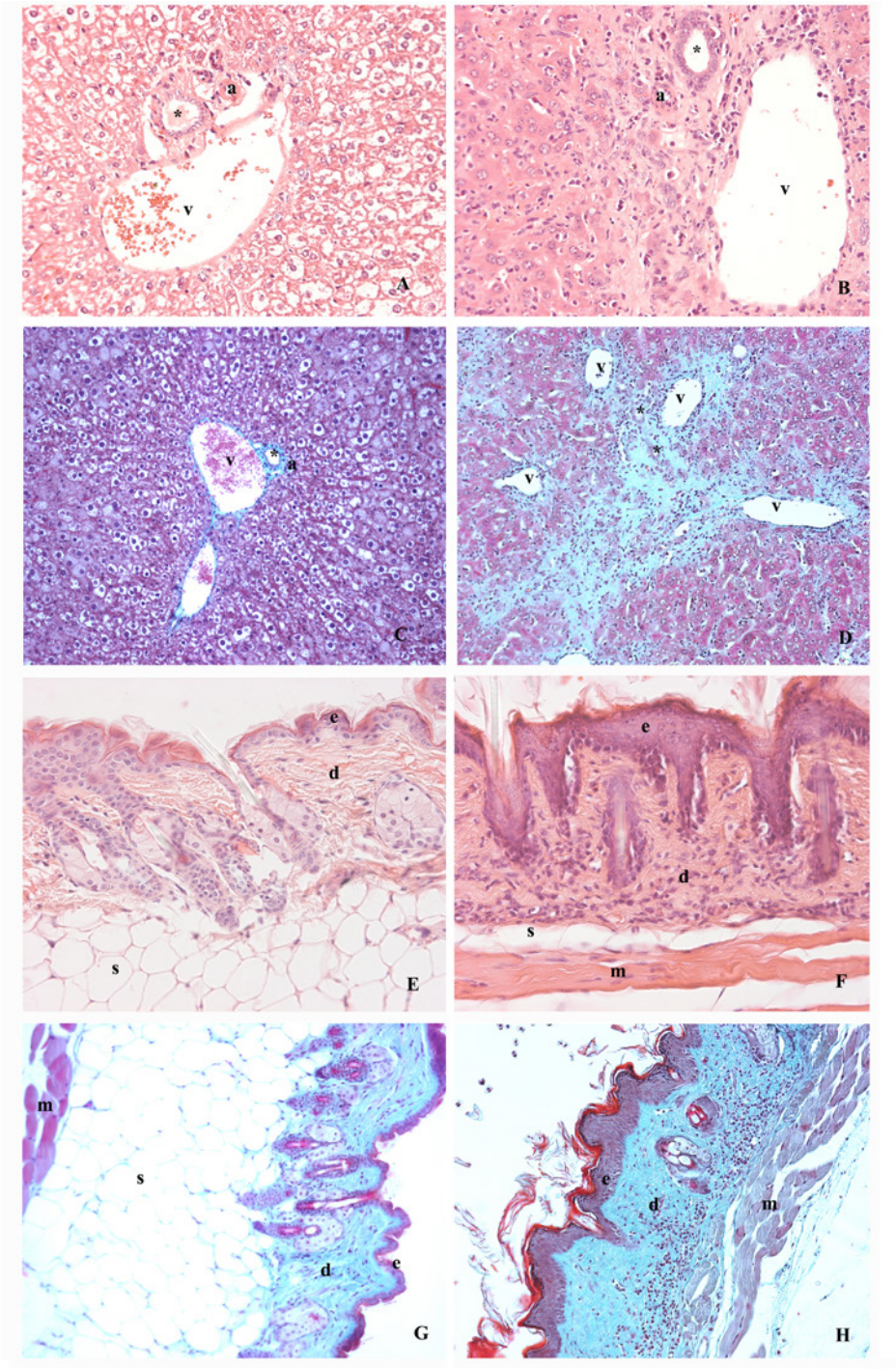
The immunohistochemical staining showed no cross-reactivity between the species, considering both primary and secondary antibodies, and were therefore considered appropriate for determining the topographic location of the different cells in the murine tissue.

As expected, no infiltrating human cells were found in the control RAG2$^{-/-}$γc$^{-/-}$ group. Only mouse-macrophages were observed in control mice tissues, using anti-mouse CD68.

In mice injected with human cells, most of the human cell infiltrate was found in the spleen, lungs and liver, but hardly any infiltrate was encountered in skin, gut, kidney, the heart and bone marrow. The different organs showed typical, consistent infiltration-patterns, which are described below. In general, most of the infiltrate concentrated in areas that would normally contain lymphoid tissue in non-immunodeficient mice and humans, and around blood vessels. None of the organs showed human macrophages, but mouse macrophages (mouse-CD68 positive) were found, mixed with the human infiltrate, mainly in spleen, lungs and liver.

Spleen. In the spleen (figure 1), the infiltrating human cells first appeared around blood vessels (day 29) and with longer survival spread diffusely in the parenchyma, with expansion of the infiltrate. The infiltrate consisted of a combination of huCD2 positive T cells (predominantly CD8 positive cytotoxic T cells (CTL's)), with B cells (huCD20 and huCD79a positive) and a considerable number of plasma cells (huCD138 and VS38c positive). The B cells and plasma cells were polyclonal for hu-lambda and hu-kappa. The B cells and some plasma cells were most often found around blood vessels in a follicular pattern, in the same areas that showed fibrosis. This site corresponds to the white pulpa zone in non-immunodeficient species. T cells spread diffusely throughout the parenchyma. Hardly any Tregs (FoxP3 positive) were detected and when present, they were scattered throughout the tissue. Murine macrophages (mouse-CD68 positive) were detected scattered diffusely through the parenchyma (data not shown).

Lungs and liver. In the murine lungs and liver (figures 2 and 3), B cells and plasma cells concentrated around blood vessels as well and to some extend spread along de bronchi. Mainly larger arteries along the bronchi were surrounded by a B cell infiltrate, closely matching the areas in which fibrosis was detected histologically. T cells spread through the alveolar septae but were also detected mixed with B cells peri-vascular. Many murine macrophages were found in the alveolar spaces (figure 2).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◂ **Figure 3 (previous page).** Histological and immunohistochemical analysis of infiltrating human cells in RAG2$^{-/-}$γc$^{-/-}$ liver and skin.
Infiltrate analysis comparing a control mouse with a representative mouse surviving 29 days (liver) and 45 days (skin) after huPBMC-injection in experiment 1. The displayed morphology is representative for the observed fibrosis and cell infiltration patterns in a total of 15 examined mice (6 mice in experiment 1 and 9 mice in experiment 2).
A. Normal murine liver, showing no infiltrate nor fibrosis in the portal tract (H&E staining, 20x, control mouse, fibrosis score 0). B. Liver after injection with human PBMC's, showing infiltration of human cells in the portal tract with extensive fibrosis and an intact bile duct 29 days after huPBMC-injection (H&E staining, 20x fibrosis score 2). C. Corresponding area to A, showing green staining of collagenous fibers in hepatic portal area of normal mice (Trichrome staining, 20x, fibrosis score 0). D. Corresponding area to B showing extensive collagenous fibrosis in green, in a mouse injected with huPBMC's (Trichrome staining 10x, fibrosis score 2). E. Normal murine skin, with many hair follicles in the dermis and normal presence of subcutaneous fat (H&E staining, 20x, control mouse, fibrosis score 0). F. Skin 45 days after injection with human PBMC's, showing extensive fibrosis of the dermal collagen at the cost of the subcutaneous fat and infiltration of human cells sub-epidermal, peri-follicular and interstitially (H&E staining, 20x, fibrosis score 4). G. Corresponding area to picture E showing a normal ratio between dermal collagen (green) and subcutaneous fat (Trichrome staining, 20x, fibrosis score 0) H. Corresponding area to picture F showing extensive collagenous fibrosis (green) in the dermis with loss of subcutaneous fat (Trichrome staining, 20x, fibrosis score 4).
a: portal artery, v: portal vein, asterisk: bile duct, s: subcutaneous fat, d: dermal tissue, e: epidermal layer, m: muscle.

In the liver, the major sites of infiltration were the peri-portal and peri-central area. B cells were located around vessels, mainly the peri-portal and peri-central located arteries (figure 3). These areas closely matched the areas of fibrosis in the previous histological analysis. T cells were located in the hepatic sinuses, together with an increased amount of murine CD68 positive macrophages. There was a mixture of T-helper cells (CD4 positive) and CTL's (CD8 positive) in both lungs and liver, without predilection for either cell-type. Only a few Tregs were detected, but they were not confined to a specific area in the tissue.

Skin. The skin showed only small numbers of infiltrating T cells (predominantly CD8 positive CTL's), sub-epidermal and surrounding hair follicles. Sporadically a single Treg could be detected, but no B cells or plasma cells were identified (figure 3). There was no correlation between the location of the previously described fibrosis and the cellular infiltrate.

Gut. The gut (including colon and ileum) showed infiltration of T cells with scattered Tregs in the submucosa, in areas where one would normally expect the mucosal associated lymphoid tissue in non-immunodeficient species. In the colon the infiltrate consisted mainly of T-helper cells (CD4 positive). In the ileum there was a relatively large amount of CTL's (CD8 positive). Like in the skin, no B cells or plasma cells were observed.

Kidneys. The kidneys contained a mild infiltrate of T cells, with a combination of CTL's (CD8 positive) and T-helper cells (CD4 positive), especially around blood vessels and sometimes near or in glomeruli.

Heart. In the heart just a few T cells were detected within major heart valves, but they could not be further specified. No human cells were encountered in the myocardium.

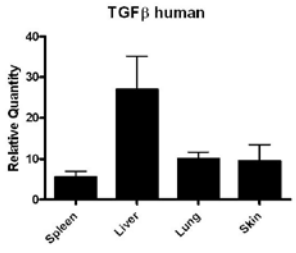
Bone marrow. The cellular bone marrow of the mice showed only few scattered human cells, consisting mainly of T cells (predominantly CD8 positive) and a sporadic plasma cell, together with a few murine macrophages. There were no human B cells and virtually no Tregs present. The increase of the reticulin fibres could not be related to a specific human cell-type in these areas.

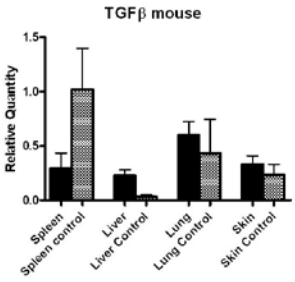
Cytokine profile

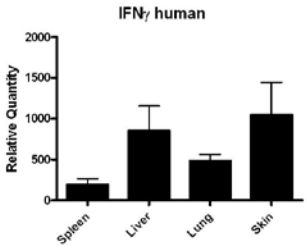
Q-PCR showed high RNA levels for the human cytokines TGF-β and especially human IFN-γ in all organs tested. The calculated relative quantities (RQ) for TGF-β were particularly high in the liver, when compared to spleen, lung, skin and gut (figure 4). The data showed a mixture of infiltrate in all affected organs, containing RNA for cytotoxic T cells (CD8) and T-helper cells (CD4), including Th1 cells (Tbet), Th2 cells (GATA3), showing some variation for the amount of these different cell types between the organs. Also Th17 cells (RORC) were present, but Tregs (Foxp3) were only observed in spleen, lungs and skin (figure 5). The detected variation for cell types, based on Q-PCR markers, correlated well with the previous immunohistochemical results. The expression of CXCR4 explained the attraction for lymphocytes to these organs.

Furthermore, only mouse-derived CD68 positive macrophages were detected. Ct values for human CD68 were very low, implicating the presence of hardly any human macrophages (figure 4).

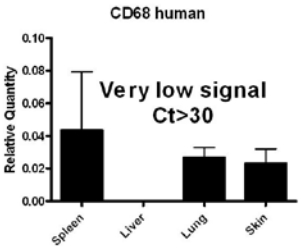
No fibrotic and other inflammatory-related cytokines: such as FGF2, PDGF, EGF (data not shown), nor BMP4 or CTGF mRNA of human origin were detected (figure 4). Only low expression of human RNA for PAI was shown in lung and very low in the skin (data not shown). However, high levels of murine CTGF were found in the lung and skin (figure 4). High levels of murine TGF-β were also observed, but this appears not to be related to human PBMC-injection, as the level of murine TGF-β in control RAG2$^{-/-}$γc$^{-/-}$mice was equally high or even higher. Low levels of murine BMP4 could be recovered from lung, skin, colon and ileum, but was also detected in both test and control mice.

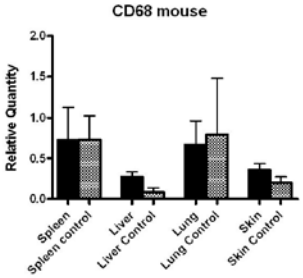




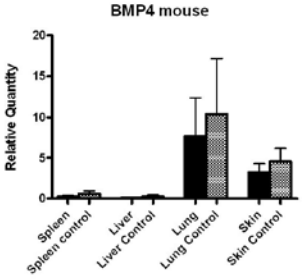


IFNy mouse
Not detectable

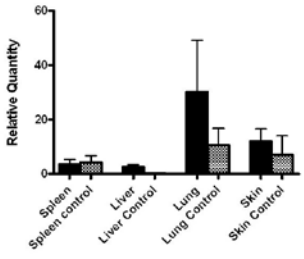




BMP4 human
Not detectable

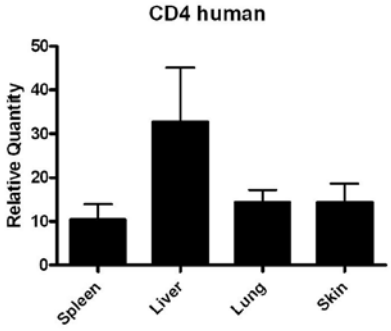


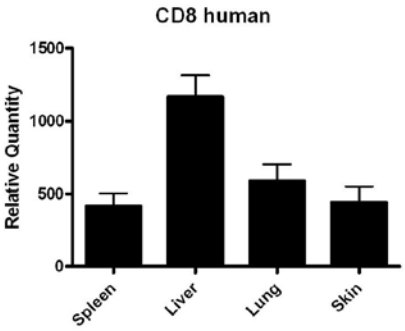
CTGF human
Not detectable

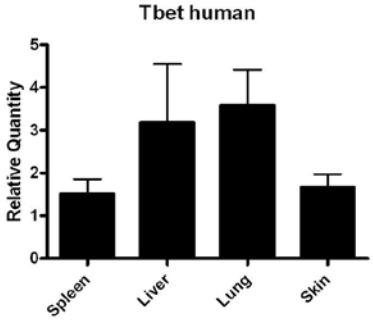


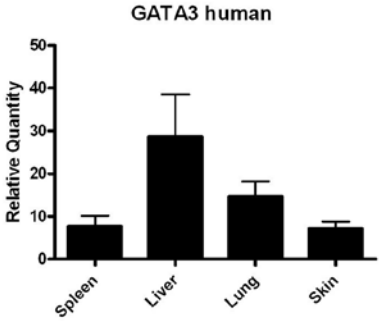
◂ **Figure 4 (previous page).** Expression of mRNA for fibrogenic proteins and macrophage markers. Relative Quantity of RNA for human and murine TGF-β, CD68, BMP4 and CTGF in spleen, lungs, liver and skin of $RAG2^{-/-}\gamma c^{-/-}$ mice injected with huPBMC's.

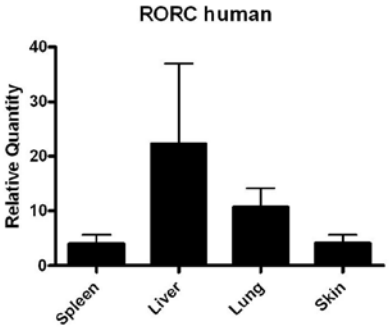
Figure 5 (next page). Expression of T cell marker mRNA. Relative Quantity of RNA for different human cell types, including FoxP3 (Tregs), Gata3 (Th2 cells), Tbet (Th1 cells), RORC (Th17 cells), CD4 (T-helper cells) and CD8 (cytotoxic T cells) including CXCR4 (chemotactin for lymphocytes) in spleen, lungs, liver and skin of $RAG2^{-/-}\gamma c^{-/-}$ mice injected with huPBMC's. ▸

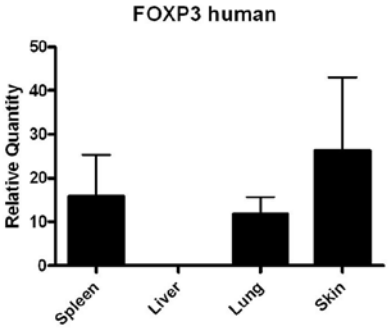

CD4 human
Relative Quantity
50
40
30
20
10
0
Spleen
Liver
Lung
Skin

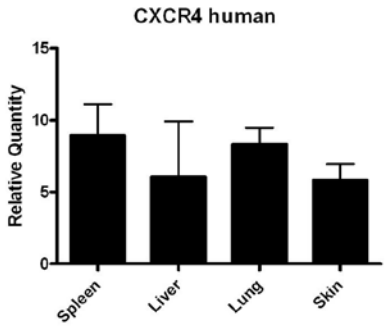

CD8 human
Relative Quantity
1500
1000
500
0
Spleen
Liver
Lung
Skin


Tbet human
Relative Quantity
5
4
3
2
1
0
Spleen
Liver
Lung
Skin


GATA3 human
Relative Quantity
50
40
30
20
10
0
Spleen
Liver
Lung
Skin


RORC human
Relative Quantity
40
30
20
10
0
Spleen
Liver
Lung
Skin


FOXP3 human
Relative Quantity
50
40
30
20
10
0
Spleen
Liver
Lung
Skin


CXCR4 human
Relative Quantity
15
10
5
0
Spleen
Liver
Lung
Skin

DISCUSSION

In this paper we report for the first time the exact, detailed aspects of sclerotic GvHD in severe immunodeficient RAG2$^{-/-}$γc$^{-/-}$ mice injected with huPBMC's, with a major focus on the morphology of the damaged organs, infiltration patterns and the molecular aspects concerning specific cytokines produced in the affected organs.

In human GvHD, acute GvHD (aGvHD) is defined based on temporal criteria as a reaction occurring within 100 days post-transplant and chronic GvHD (cGvHD) developing 100 days after transplantation. In humans, a small subset of these patients with cGvHD develop a sclerodermatous GvHD (ScGvHD) with fibrotic changes. The time-based distinction between human acute and chronic GvHD however cannot be translated to mouse-models, as no murine model for GvHD has kinetics similar to human. Most mouse models show lethal GvHD within 80 days or less. This would imply, that GvHD studies in mice might better be evaluated based on their symptomatic similarities to human aGvHD and cGvHD (for example the ScGvHD subtype), as opposed to time of onset. The distinction between aGvHD and cGvHD in mice is either made based on predominant T cell subset (Th1 in aGvHD and Th2 in cGvHD) and/or the presence of for example autoantibody production and systemic fibrosis [29]. Since our Q-PCR results show that both Th1 and Th2 cells were present, this could not be used as differentiation criterion in our model. The presence of a systemic fibrotic reaction suggests that in our model, a consistent chronic reaction occurred that looks similar to ScGvHD. As the mice that we used are immunodeficient and therefore the autoimmune component of human chronic GvHD mediated by autoaggressive T cells emerging from damaged thymus is not seen in this model, we did not test for the presence of autoantibodies. The sclerotic response in our model is exclusively mediated by mature peripherally-derived donor T cells.

Several humanized mouse models, including NOG [21], NOD/SCID [21], NOD/SCID IL2rγ-null [29], BALB/c-RAG2$^{-/-}$IL2rγ$^{-/-}$ [21, 29] or NOD/SCIDβ2m-null [23, 29] as well and mouse-mouse models, for example D10.D2 injected BALB/c mice [30-32], have been reported as models to study the diverse aspects of GvH responses. These studies report several histological features of a GvH reaction with sclerotic changes, such as the presence of lung fibrosis [21, 23, 29, 32], skin fibrosis [21, 23, 30] and fibrotic changes in the liver [21, 23, 31]. Nevertheless, an exact morphological description of these and other tissues involved in our human-mouse model was lacking. Furthermore, no specific infiltration patterns for the different human cell-phenotypes were described until now for neither xenogeneic mouse models nor mouse-mouse models.

In our model, human cells infiltrated especially the spleen, liver and lungs. Only low quantities of human cells were detected in the skin, colon, ileum and kidney and sporadically in heart valves and bone marrow. This correlates with the known sites of infiltration in other xenogeneic models [21, 23]. The infiltrate showed a mixture of CTL's and T-helper cells (including Th1, Th2- cells and Th17-cells), B cells and plasma cells, based on immunohistochemistry and Q-PCR data. The human cells localize in areas that in non-immune deficient species would contain lymphoid tissue (in spleen and gut). However, in the lungs and liver the expected bronchial associated lymphoid tissue was not involved and infiltrating B cells were often found around blood vessels. The localisation of B cells was confined to spleen, lungs and liver. The T cells, including the minute amounts of Tregs, were found diffusely spread throughout the parenchyma or in the lymphoid origins. Only murine macrophages (mouse-CD68 positive) were encountered in spleen, liver, lung, skin and gut, but no human-CD68 positive macrophages were detected. The organs showed various degrees of fibrosis, in which the severity of the fibrosis correlated with the survival time of the mice. Collagenous fibres were found around blood vessels in almost all cases, but could increase and destroy the full architecture of an organ in mice with a longer lifespan, correlating with a higher infiltration of human cells in these mice.

As emphasized by Chu *et al* [33] in a review concerning mouse-models for GvHD, mouse-models might not exactly match/mimic clinical GvHD, but they do teach us many aspects of the complex mechanism of cGvHD, including ScGvHD and are therefore very useful.

Our data show that the morphology of the skin reaction in our model is similar to the one described in detail in other mouse-mouse models [30] and xenogeneic models [21, 23], and is similar to the human skin reaction in clinical ScGvHD. This includes the paradox of low human cell infiltrate with nevertheless evident fibrosis. Many studies try to relate this effect to either CD4 or CD8 positive T cells. Although the GvHD effect in immunodeficient mice is considered to be predominantly mediated by CD4 positive cells [34, 35] some studies report the importance of CD8 positive cells in the epidermis in both aGvHD and cGvHD skin biopsies [36] and oral cGvHD [37]. In our humanized mouse-model most T cells were CD8 positive. However, this does not explain the consequent low cell infiltrate. Although the amount of T cell infiltration in the skin was low compared to spleen, lungs and liver, the production of TGF-β was not significantly different from the TGF-β production in spleen or lung as well as the IFN-γ production. This underscores the possibility of a direct role of cytokines in inducing tissue damage as suggested by Dickinson *et al*. [38], independent of the quantitative amount of human cell infiltrate.

In contrast to human GvHD and other xenogeneic models, the gut of the RAG2$^{-/-}$γc$^{-/-}$ mice showed no signs of damage at all, especially no signs of apoptotic colitis, collagen deposition or any other gastro-intestinal features that one would normally expect in human aGvHD and chronic forms of GvHD. This might be related to differences in intestinal bacterial microflora in these SPF bred mice. The intestinal flora is considered to have an important role in the tuning of T cell repertoire in gut-associated lymphoid tissue [39], although the exact relation to GvH response remains unclear.

The pathology in the lung is compatible to what is observed in other xenogeneic models and is to some extend comparable to human ScGvHD, in which interstitial fibrosis has been described as a bronchiolitis obliterans pattern [40], but instead of concentrated along bronchioles, it merely aligned blood vessels in our mice. Chronic human GvHD in the liver, though rarely biopsied, shows either the classic signs of bile duct epithelial injury with portal lymphocytic infiltration, or the hepatic variant with relatively mild bile duct injury and lobular hepatitis [41]. Nevertheless, the loss of bile ducts in human cGvHD might occur only late in the reaction [42, 43] and clinical cGvHD may mimic a variety of autoimmune and immunodeficient diseases [33]. The liver morphology in our model did show some resemblance to the histology of human autoimmune hepatitis, including the periportal fibrosis mentioned by Howell *et al* [31].

Several cytokines are known to be involved in the GvH reaction, including IFN-γ and TGF-β [44]. Our Q-PCR data showed production of both human and murine cytokines (figures 4 and 5). The high levels for TGF-β that we detected in our model are in line with the development of fibrosis in general [45-47]. CTGF is a TGF-β inducible early gene in fibroblasts [48] and directly interacts with BMP4 and TGF-β ligands in extra cellular spaces. CTGF has been found to correlate with the formation of sclerosis in scleroderma [49, 50] but has not been reported in the context of ScGvHD [51]. In our model high levels of murine-CTGF were detected, which can only be produced by non-lymphoid murine cells, for the lack of RAG2 and the γc-alleles prevented the formation of murine B cells, T cells and NK cells. The significant high levels of human TGF-β could only be produced by human B cells and human T cells, for human macrophages were lacking in our model. Murine TGF-β and murine-BMP4 were elevated in both injected mice and control mice and this high level of TGF-β mRNA and therefore cannot be directly related to the development of GvHD by huPBMC's. No expression of other fibrosis related factors of murine or human origin were detected. As IFN-γ may have various activities [52-55], the implications of IFN-γ in our process cannot easily be explained, although it has been related to the fibrosis observed in chronic allograft vasculopathy [56].

Interplay of donor cells with host derived antigen presenting cells, to which macrophages belong, and a monocyte driven fibrotic reaction in the skin has been suggested in the past [32, 33]. Based on our results, we conclude that there must be interplay between the human B and T cells and mouse macrophages at the level of human TGF-β and IFN-γ with murine CTGF, resulting in the formation of fibrosis.

Despite some differences with human cGvHD, more specifically the human ScGvHD subtype, we must agree with Chu *et al* and Schroeder *et al* [29, 33] that mouse-models are crucial for GvHD studies, although "one should choose their mouse-model wisely" based on the targets of interest. Apart from differences in GvH phenotype between different mouse-strains, even in the same model for GvH response, the outcome of the induced reaction often depends on conditioning regimen, irradiation dose, donor source or even age of the transplanted mice [29]. In our experiments the level of human chimerism varied and some variation was observed when comparing the scores of the histological or immunohistochemical evaluation for significance. Nevertheless, we were able to show a consistent pattern, either significant or at least as a trend, for both morphology and migration in our human-mouse model. We therefore consider detailed histopathological features, including the similarities and differences between mouse-models and human cGvHD (more specifically ScGvHD), an absolute necessity for future studies considering GvHD.

In conclusion, our histological, immunohistochemical and molecular analysis of the ScGvHD in the RAG2$^{-/-}$γc$^{-/-}$ mouse, establishes this human-mouse model as relevant to study ScGvHD and it has enabled us to dissect the various processes that are involved in GvHD and are caused by either donor or recipient cells. It now opens the possibility to specifically study therapeutic approaches directed to either of these processes using reagents directed to either mouse or human targets.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no competing financial interests.

SUPPLEMENTARY DATA

Table S1. Individual histopathology scores for spleen, liver lung and skin in experiment 2.

			Histopathology score				
Organ	**Mouse group**	**Mouse ID**	**Fibrosis**	**T cells**	**B cells**	**Plasma cells**	**Tregs**
Spleen	HuPBMC	1	3	4	3	1	0
		2	2	4	3	2	2
		3	2	4	3	2	0
		4	2	4	3	4	0
		5	3	4	2	3	0
		6	0	4	3	3	2
		7	0	3	2	2	3
		8	3	3	3	2	1
		9	1	3	0	0	3
	Control RAG2-/- γc-/-	C1	1	0	0	0	0
		C2	1	0	0	0	0
		C3	1	0	0	0	0
		C4	1	0	0	0	0
		C5	1	0	0	0	0

			Histopathology score				
Organ	**Mouse group**	**Mouse ID**	**Fibrosis**	**T cells**	**B cells**	**Plasma cells**	**Tregs**
Lung	HuPBMC	1	3	3	1	1	1
		2	2	3	0	0.5	1
		3	2	3	0	0	1
		4	3	2	0	0	1
		5	4	3	2	2	0
		6	1	2	1	2	1
		7	2	3	1	0	1
		8	1	2	0	0	0
		9	1	3	1.5	1	1

			Histopathology score				
Organ	**Mouse group**	**Mouse ID**	**Fibrosis**	**T cells**	**B cells**	**Plasma cells**	**Tregs**
Liver	HuPBMC	1	2	2	0.5	0	0
		2	3	2	0	0	1
		3	2	0	0.5	0	1
		4	1	2	1	0.5	0
		5	2	3	1	1	1
		6	1	0	1	0.5	1
		7	1	3	1	0.5	0
		8	2	3	1	0.5	0
		9	1	3	1.5	1	1
	Control RAG2-/- γc-/-	C1	0	0	0	0	0
		C2	0	0	0	0	0
		C3	0	0	0	0	0
		C4	0	0	0	0	0
		C5	0	0	0	0	0

			Histopathology score				
Organ	**Mouse group**	**Mouse ID**	**Fibrosis**	**T cells**	**B cells**	**Plasma cells**	**Tregs**
Skin	HuPBMC	1	1	3	0	0	0
		2	2	3	0	0	1
		3	1	1	0	0	1
		4	1	1	0	0	0
		5	3	4	0	0	0
		6	2	2	0	0	0
		7	3	3	0	0	2
		8	1	1	0	0	0
		9	2	2	0	0	0

Table S1. Continued

			Histopathology score				
Organ	**Mouse group**	**Mouse ID**	**Fibrosis**	**T cells**	**B cells**	**Plasma cells**	**Tregs**
Lung	Control RAG2-/- γc-/-	C1	0	0	0	0	0
		C2	0	0	0	0	0
		C3	1	0	0	0	0
		C4	0	0	0	0	0
		C5	0	0	0	0	0

Individual histopathology scores for fibrosis, T cells, B cells, plasma cells and Tregs in spleen, liver, lung and skin for RAG2$^{-/-}$γc$^{-/-}$ mice injected with huPBMC and control RAG2$^{-/-}$γc$^{-/-}$ mice in experiment 2. Severity of infiltrate, with respect to B cells, T-helper cells, cytotoxic T cells and plasma cells was scored in a scale of 0-4, as no infiltration (score 0), sporadic / less than 5% infiltration (score 1), mild infiltration varying between 5-25% (score 2), moderate infiltration of 25-50% (score 3) or severe infiltration of more than 60% (score 4).

			Histopathology score				
Organ	**Mouse group**	**Mouse ID**	**Fibrosis**	**T cells**	**B cells**	**Plasma cells**	**Tregs**
Skin	Control RAG2-/- γc-/-	C1	0	0	0	0	0
		C2	0	0	0	0	0
		C3	0	0	0	0	0
		C4	0	0	0	0	0
		C5	0	0	0	0	0

Since the presence of Tregs in human blood varies from 1-10%, different thresholds had to be used to score the presence of human Tregs: no infiltration (score 0), sporadic infiltration of about 1% (score 1), mild infiltration of about 3-5% (score 2), moderate infiltration of more than 5-7% (score 3), severe infiltration of more than about 7% (score 4). Scores represent the final scores of both pathologists together (Krippendorffs's kappa score 0.6), in which intermediate scores could be given between two consecutive scores when agreement could not otherwise be achieved.

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CHAPTER 4

MODIFYING GRAFT VERSUS HOST DISEASE IN A HUMANIZED MOUSE MODEL BY TARGETING MACROPHAGES OR B CELLS

Marieke C.H. Hogenes[1,2], Suzanne van Dorp[3], Joyce van Kuik[2], Filipa R.P. Monteiro[2], Natalie ter Hoeve[2], Liane Guedes[2], Marijke R. van Dijk[2], Anton C. Martens[4], and Roel A. de Weger[2].

[1]Laboratory for pathology East Netherlands,
[2]Department of Pathology UMC Utrecht,
[3]Department of Hematology (DIGD) UMC Utrecht,
[4]Department of Hematology, VU University Medical Centre, Amsterdam

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ABSTRACT

Humanized mouse models can well be modified to study specific aspects of graft-versus-host disease (GvHD). This paper shows the results of both macrophage depletion and (early) B cell depletion in a humanized mouse model using RAG2$^{-/-}$γc$^{-/-}$ mice injected with HuPBMC's.

Macrophage depletion showed a significant decrease in survival, and also lead to a change in the histomorphology of the xenogeneic reaction. Higher levels of infiltrating B cells were observed in various organs of mice depleted for macrophages.

With (early) B cell depletion using Rituximab, a clear improvement on clinical symptoms was observed, even when probably only inactivated B cells were deleted. However, the histological examinations only showed a significant morphological effect on liver fibrosis. This may be related to a difference in mRNA levels of TGF-β. Also lower mRNA levels of Tregs in some organs were observed after Rituximab treatment, which contradicts that a higher number of Tregs would always be related to less severe GvHD.

Our data show that both macrophage depletion as well as (early) B cell depletion in a xenogeneic mouse model can influence clinical, histological and cytokine production of a GvHD response.

INTRODUCTION

Since researchers developed an interest in the pathogenesis and possible treatment targets of human graft-versus-host Disease (GvHD), the interest in mouse models has grown. Several mouse models, including humanized mouse models, are available as discussed in our previous paper [1] with endless options to modify the model to your needs. One of these models is the RAG2$^{-/-}\gamma c^{-/-}$ mouse model [1], in which the immunodeficient mice lack mature T cells, mature B cells and NK cells, but maintain monocytes and macrophages.

The list of interesting research targets in GvHD is long and each step takes us closer to unraveling the complex reaction occurring after bone marrow transplant and donor lymphocyte infusion. In early days one thought that cytotoxic T cells were involved, today's knowledge shows an important role in GvHD for regulatory T cells (Tregs) and B cells as well. Tregs have a suppressive effect by down-regulating the proliferative activity of T cells [2]. For B cells, a positive influence on GvHD activity has been noted when using Rituximab, a B cell depleting antibody [3-6]. Rituximab was originally developed to treat Non-Hodgkin lymphomas [4, 7], but is successful in a variety of autoimmune diseases as well [8].

A possible interaction between Tregs and B cells cannot be excluded. Previous research has shown an association of B cell depletion with a change in numbers of CD4+ and CD8+ T cells, as well as changes in their activation status [9]. Early phase B cell depletion is associated with prominent increased mRNA levels of genes characterized by Tregs in Rituximab-treated patients, including CD25, FoxP3, CTLA-4, GITR and TGF-beta [10]. Rituximab treatment reduces CD4+ T-cells with expression of the costimulatory/ activation molecule CD40L [10]. Some studies suggested that the effect of B cells on Tregs might be activation depended [11-13], others suggest that Tregs directly influence B cells due to their presence not only in the paracortical zone but also in the mantle-zone of a follicle [14] .

We have noticed a difference in methods in both Tregs en B cell studies, in which some researchers have used clodronate depletion to accompany the mouse conditioning regiment and some have not. Clodronate is a pharmakon associated with induction of apoptosis in monocytes, macrophages and osteoclasts [15-19]. The half-life of clodronate is very short when released in circulation and it does not easily cross cell membranes. However, to avoid this issue one could embed clodronate in multilamellar liposomes, which are exclusively ingestible for phagocytic cells [20]. Once ingested, clodronate accumulates within macrophages, resulting in a toxic level of clodronate that ultimately leads to apoptosis of the macrophages [21]. After clodronate depletion therapy, the number and phenotype of the residual of any given tissue depends on the tissue

being examined and will change during infection of inflammation [22]. Similarly, the ability to deplete macrophages using this protocol will depend on the route of administration. Intravenous injection for example results in depletion of macrophages from mainly spleen and liver within one day after single dose administration [20, 23]. Repopulation of these macrophages in the liver starts as soon as 5 days post-injection [24] and within a week in spleen [25]. However, it cannot be excluded that donor macrophages might also be depleted with this protocol, as van Rijn *et al*. showed that donor CD14+ cells disappear soon after huPBMC injection when clodronate had been administered [26].

Even though it is known that different pre- and post-treatments in your humanized mouse model will have a direct effect on the severity of the displayed symptoms, less is known about the effects of depleting specific cell lines in the modification of a humanized mouse model that might influence the resulting type of reaction and its histomorphology or cytokine profile.

In this paper we take a closer look on the effect of depleting host-macrophages and donor-B cells on tissue morphology, as well as the resulting cytokine profile in the xenogeneic RAG2$^{-/-}$γc$^{-/-}$ mice mouse model.

MATERIALS AND METHODS

Mice and conditioning regiments

RAG2$^{-/-}$γc$^{-/-}$ mice were obtained from the Netherlands Cancer Institute (Amsterdam, the Netherlands) [27]. The mice were bred and maintained under micro-isolator cages under specified pathogen-free conditions at the Central Laboratory Animal Institute (Utrecht University, The Netherlands) and were given sterile water and irradiated pellets ad libitum. All experiments were conducted after permission was granted by the local Ethical Commit- tee for Animal Experiments and in accordance with the Dutch law on animal experimentation.

Macrophage depletion model

Macrophage depletion was studied in duplo, using the same conditions described by van Rijn *et al*. [26]. In both experiments, all mice were given total body irradiation (TBI) with a single dose of 350 cGy (gamma irradiation from a linear accelerator) prior to their further conditioning for the study.

The first experiment (referred to as experiment 1) used a total of 20 female mice (n=20), in three groups. The macrophage-depletion group (n=5) received intravenous clodronate containing liposomes injection just after their TBI, followed by the intravenous injection of 30x10^6 human

peripheral blood mononuclear cells (huPBMC's) the day after. The non-macrophage-depleted group (n=10) only received huPBMC's the day after TBI. The mice in the control group (n=5) were irradiated, but did not receive huPBMC's nor clodronate.

The second experiment (referred to as experiment 2) used 16 male mice (n=16), in two groups. The macrophage-depleted group (n=8) received clodronate containing liposomes after TBI, followed by intravenous injection of huPBMC's from 2 different donors the following day (containing at least $10x10^6$ T-cells: $6x10^6$ huPBMC's for donor A and $10x10^6$ huPBMC's for donor B) and the non-depleted group (n=8) only received these huPBMC's the day following TBI.

Mice were monitored for symptoms of GvHD as described previously [28]. Mice were sacrificed by cervical dislocation after 70 days or when losing more than 20% of their original body weight or when they experienced severe symptoms, wound infection of signs of bleeding caused by the given injections.

Preparation of clodronate containing liposomes

Clodronate-containing liposomes were prepared as described by van Rijn *et al* [26]. Briefly, a mixture of egg-phosphatidylcholine, egg-phosphatidylglycerol (both from Lipoid, Ludwigshaven, Germany) and cholesterol (Sigma, StLouis, MO) were dissolved in ethanol in a molar ratio of 10:1:1.5 and evaporated to dryness by rotation under reduced pressure. The lipid film was hydrated in an aqueous solution containing clodronate (Cl2MDP, concentration 60 mg/mL, Bonefos; Schering, Weesp, the Netherlands). The unencapsulated clodronate was removed by repeated washing using PBS pH 7.4 and ultracentrifugation (Beckman Optima LE-80K; Palo Alto, CA) at 200,000g for 30 min. After washing, the pellet was resuspended in PBS at a concentration of 90 mM phospholipids. The final concentration of clodronate was determined using spectrophotometry at a wavelength of 238 nm after extraction and binding to Cu^+ and appeared to be within a range of 2-2.5 mg/ml. Mice prone for depletion of macrophages, received 0.2 mL of the liposome-suspension intravenously one day before injection of huPBMC's.

B cell depletion model

B cell depletion was studied, using 30 female $RAG2^{-/-}\gamma c^{-/-}$ mice, separated in 4 groups. All mice received TBI one day prior to further conditioning as described below. Injection of Rixtuximab and/or human cells was performed intravenously. One group of mice (n=10) was injected with 50 mg Rituximab followed by 7.5-15 $x10^6$ huPBMC's. The second group (n=10) was injected with huPBMC's only, the third group (n=5) with 50 mg Rituximab only and a fourth group (n=5) received TBI only.

Mice were monitored for development of symptoms of GvHD as described previously [28] and sacrificed by cervical dislocation either after 70 days or when either losing more than 20% of their original body weight, having severe symptoms, wound infection or bleeding because of the given injections or when they experienced side effects of the medication. FACS analysis with a FACSCalibur (Becton Dickinson) was performed to evaluate the fraction of human cells in the murine peripheral blood, using human CD45 (huCD45), mouse CD45, huCD3 and huCD19 (Becton Dickinson), as described by van Rijn *et al.* [26]

Preparation and transplantation of huPBMC's (both models)

HuPBMC's were prepared as described previously [26]. Briefly, fresh buffy coats were obtained from healthy human volunteers at the Bloodbank of the University Medical Centre Utrecht and huPBMC's were isolated using Fiqoll Hypaque (Pharmacia, Uppsala Sweden) density centrifugation. Cells were washed twice in phosphate-buffered saline (PBS) and resuspended in PBS/0.1% human serum albumin (HSA). Fresh cell suspensions of 0.2 ml, (containing huPBMC's in concentration as stated above and using a different human donor in each experiment) were injected intravenously in the tail vein, without prior ex-vivo stimulation.

Histology and immunohistochemistry (both models)

In general

Autopsy was performed on all mice in both the macrophage-depletion model as well as the B cell depletion model. Spleen, lungs, liver, ileum, colon, skin (from cervical region) and femur were collected for further analysis. Each organ was split (femur split as being right sided and left sided); one part was frozen and stored in liquid nitrogen and another part was fixed in PBS-buffered formaldehyde (4%). The femur was decalcified with EDTA. The formalin fixed organs were embedded in paraffin and tissue sections were cut at 4 µm using coated slides, for staining with haematoxylin-eosin (H&E) for histology and several primary antibodies for immunohistochemistry (Appendix A in supplement section) as described previously [28] . In our second macrophage-depletion study, tissue microarray blocks (TMA) were made.

Immunohistochemical staining was performed on slides from FFPE-blocks in a Bond-maxTM automated immunostainer (Leica Microsystems, United Kingdom) after pre-treating automatically with either citrate, using the Bond Epitope Retrieval solution 1 (AR9961), or EDTA, using the Bond Epitope Retrieval solution 2 (AR9640) with washing steps between each reagent using 1x Bond Wash Solution. The staining was identified by a polymer based detection system with Diamino-benzidine (DAB) using the Bond Polymer Refine Detection kit (DS9800). The sections were counterstained with haematoxylin.

For human FoxP3 staining slides were deparaffinized, washed and blocked with endogenous peroxidase blocker. Antigen retrieval was performed using citrate pre-treatment; slides were then washed and incubated with diluted anti-human FoxP3 antibody for 1 hr. After rinsing, slides were incubated for 30 min with rabbit-anti-rat-HRP (DAKO, Glostrup, Denmark), diluted at 1:250, and incubated with Powervision Goat-anti-rabbit IgG HRP (Immunologic, Klinipath, the Netherlands), followed by DAB staining for 10 min after washing with PBS. Slides were counterstained with haematoxylin.

Mouse-CD68 staining was performed on frozen tissue sections cut at 8 µm and stained as described previously [28] using combination of rabbit-anti-mouse antibody-HRP (DAKO) and 10% mouse serum (DAKO) in a dilution of 1:100. Staining was identified using DAB and counterstaining with haematoxylin.

Cross-reactivity of all monoclonal and polyclonal antibodies used (against human antigens), was tested using both a positive control (human tonsil) and a double negative control. These negative controls consisted of a control staining using BALB/c tissue and of murine tissue from $RAG2^{-/-}\gamma c^{-/-}$ mice without huPBMC's. In these $RAG2^{-/-}\gamma c^{-/-}$ mice without huPBMC's, a control was run with primary antibody and a control staining using murine tissue with human cells without the primary antibody to rule out both false positive staining due to cross-reactivity of the other reagents used, and false negative staining for the primary antibody. Cross-reactivity of anti-murine CD68 was tested in human tonsil tissue.

Slides were assessed for presence, severity and location of both fibrosis and cell infiltrate, including the huPBMC's and murine macrophages by two experienced pathologists and an experienced molecular biologist using light microscopy, using our the previously described method [28].

Macrophage depletion model

In these experiments spleen, lungs, liver, ileum and colon, skin and femur were isolated from each mouse. From the first experiment only 9 mice could be used for histological analysis of all these organs (a total of n=9 per organ, n=6 for non-depletion, n=3 for macrophage depletion). From the second experiment 16 mice were available for both histological as well as cytokine analysis (adding up to n=16 per organ, n=8 for non-macrophage depletion and n=8 for macrophage depletion).

B cell depletion model

Spleen, lungs, liver, ileum and colon, skin and femur were isolated; adding up to a total of n=28 per organ available for complete analysis, separated in 4 groups (n=10 for rituximab with huPBMC's, n=9 for huPBMC's only, n=3 for Rituximab only, n=5 for neither huPBMC's nor Rituximab).

Cytokine profile analysis

The cytokine gene expression profile was analysed by quantitative polymerase chain reaction (Q-PCR) to measure the amount of human and murine messenger RNA (mRNA) in the tissues.

For macrophage depletion, RNA was isolated from all mice for spleen, liver, lung, skin and bone marrow. For B cell depletion analysis, spleen, liver, lung, skin, colon, ileum and femur were isolated and cytokine analysis was performed for several murine and human cytokines as described previously [13].

RNA was isolated using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. Of each sample 3 µg RNA was incubated with 1 µl of both oligoDT (Promega, Leiden, The Netherlands) and Random Primers (Promega) for 5 min at 70˚C. Finally, cDNA was synthesized by adding a mixture of first strand buffer 5x (Invitrogen, Breda, the Netherlands), 0,1 M DTT, 10mM dNTP's (Invitrogen) and incubation for 1 hr at 42˚C. The cDNA was stored upon use at -20˚C.

For all cytokines and other gene-targets tested, the human and mouse specificity was determined by testing the primer-probe combination against mRNA isolated from normal human and murine tissues ($RAG2^{-/-}\gamma c^{-/-}$ and BALB/c mice). Only species specific pre-developed TaqMan Assays were used (TaqMan Gene Expression Assay: Applied Biosystems, Foster City, CA). The Q-PCR reactions were run on either a LightCycler 480 real-time PCR system (Roche Diagnostics, Lewes, U.K.) or a ViiA 7 Real-Time PCR system (Thermofisher Scientific, Landsmeer, The Netherlands). Each sample was run in duplicate and a negative control was used to exclude contamination. GAPDH was used as reference gene. The relative mRNA quantity was determined using the E-method of the LightCycler 480 software (Roche) and the ViiA 7 software (Thermofisher Scientific). In this method a correction for PCR efficiency is embedded in the calculation of the relative quantity of gene expression (RQ value).

All samples were analyzed for RNA levels of human TGF-β, IFN-γ, CXCR4, as well as fibrosis related factors like FGF2, PDGF, EGF, BMP4, CTGF, and multiple cell specific factors (FoxP3, GATA3, Tbet, PAI, RORC, CD4, CD8, CD20 and CD68) in order to further specify the involved cells. Samples were also analyzed for murine markers TGF-β, CTGF, BMP4, CD68 and IFN-γ. An explanatory list of the abbreviated cytokines and markers is included in Appendix B of the supplement section.

RESULTS

Clinical symptoms

Macrophage depletion model

Both macrophage depletion experiments showed a significant difference in survival, in which survival of mice depleted with macrophages was impaired (p=0.0005 in experiment 1 and p=0.0099 in experiment 2). Kaplan-Meier curves were plotted to compare survival in both experiments (figure 1). In both experiments the mice showed severe weight loss, ruffled fur, reduced mobility, erythema of the skin and in a few splenomegaly (mostly in mice without macrophage depletion). Macrophage depletion was considered effective, as a significant difference between clodronate-treated mice and non-treated mice was determined that could not be explained otherwise. qPCR analysis for macrophages (CD68) was performed at time of death related to the initiated graft-versus-host response (see below), with mean survival of mice treated with clodronate being 13 days in our first experiment and 25.5 days in our second experiment (with mean survival after clodronate-treatment was 22 days for donor A and 27 days for donor B).

B cell depletion model

B cell depletion was considered successful as histologically, numbers of B cells were significantly reduced compared to mice not treated with Rituximab (see below). The huPBMC-injected groups showed a significant difference in survival (p=0.02), with a beneficial effect on survival after Rituximab treatment. Survival was also significantly related to the development of GvHD symptoms and developing symptoms was significantly related to the amount of huPBMC's reconstituting the mice (p<0001). Symptoms of affected mice again included severe weight loss, ruffled fur, reduced mobility, erythema and in some cases splenomegaly. The number of circulating huPBMC's appeared to be lower after Rituximab treatment (figure 2), but this did not reach statistical significance (p=0.2046).

Histomorphological infiltrate analysis

Macrophage depletion model

Infiltration patterns of cells and fibrosis in both experiments followed the known pattern for this xenogeneic model (see reference [28] about the description of this model). In our first experiment, macrophage depletion showed significant increased B cell infiltration in liver (based on CD79a), lung and femur (both based on CD20). In addition, a significant increase was found on the number of plasma cells in the bone marrow (CD138) (Table 1).

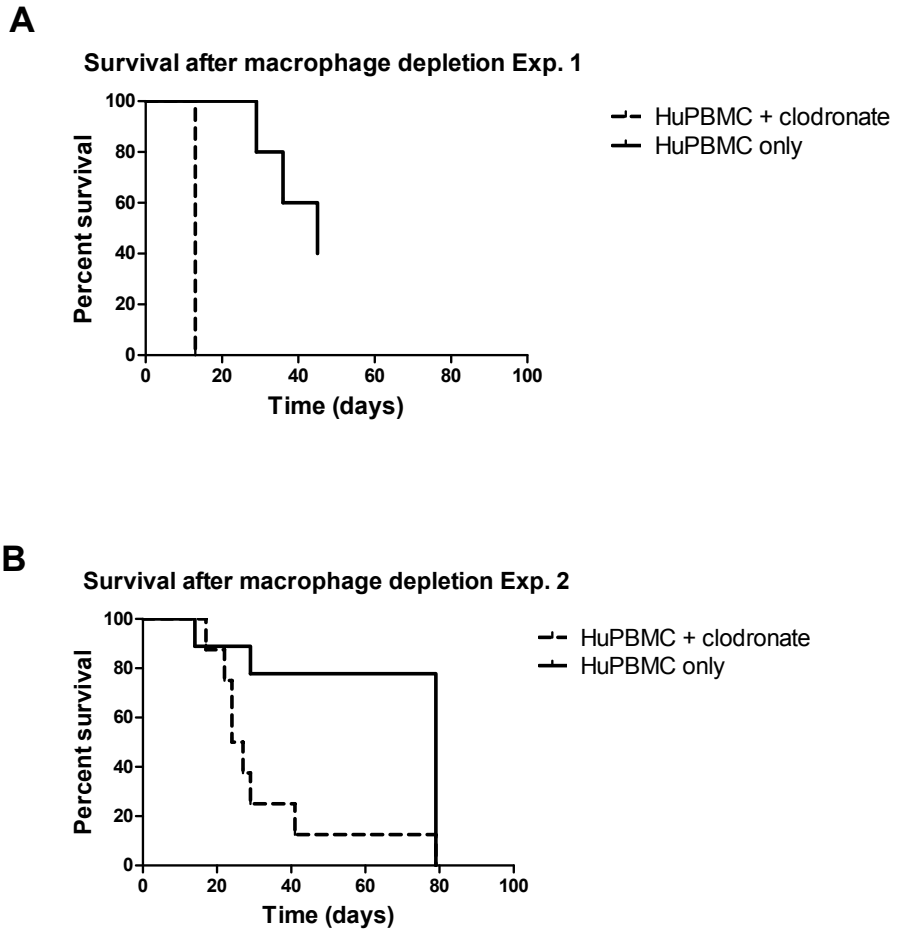

Figure 1. Survival of RAG2$^{-/-}$ γc$^{-/-}$ mice after introducing HuPBMC's with or without clodronate driven depletion of macrophages. A. Results from experiment 1. Mean survival with macrophage depletion (dotted line) was 13 days (SD 0), mean survival without macrophage depletion (continuous line) 36.67 days (SD 7.174) after intravenous injection with huPBMC's. B. Results from experiment 2. Mean survival 32.88 days (SD 19.0) with macrophage depletion (dotted line) and mean survival 64.63 days (SD 26.9) without macrophage depletion (continuous line, pooled data from both donors after huPBMC injection).

The overall analysis of the second experiment showed significant increased fibrosis in the femur, an increased number of T cells in liver, lung, skin, colon and femur, increased numbers of B cells in spleen, liver and lung as well as a variable increase or decrease in the number of plasma cells in spleen, liver and lung (Table 1), though when comparing the different donors used, this significance did not apply for all donors at all times.

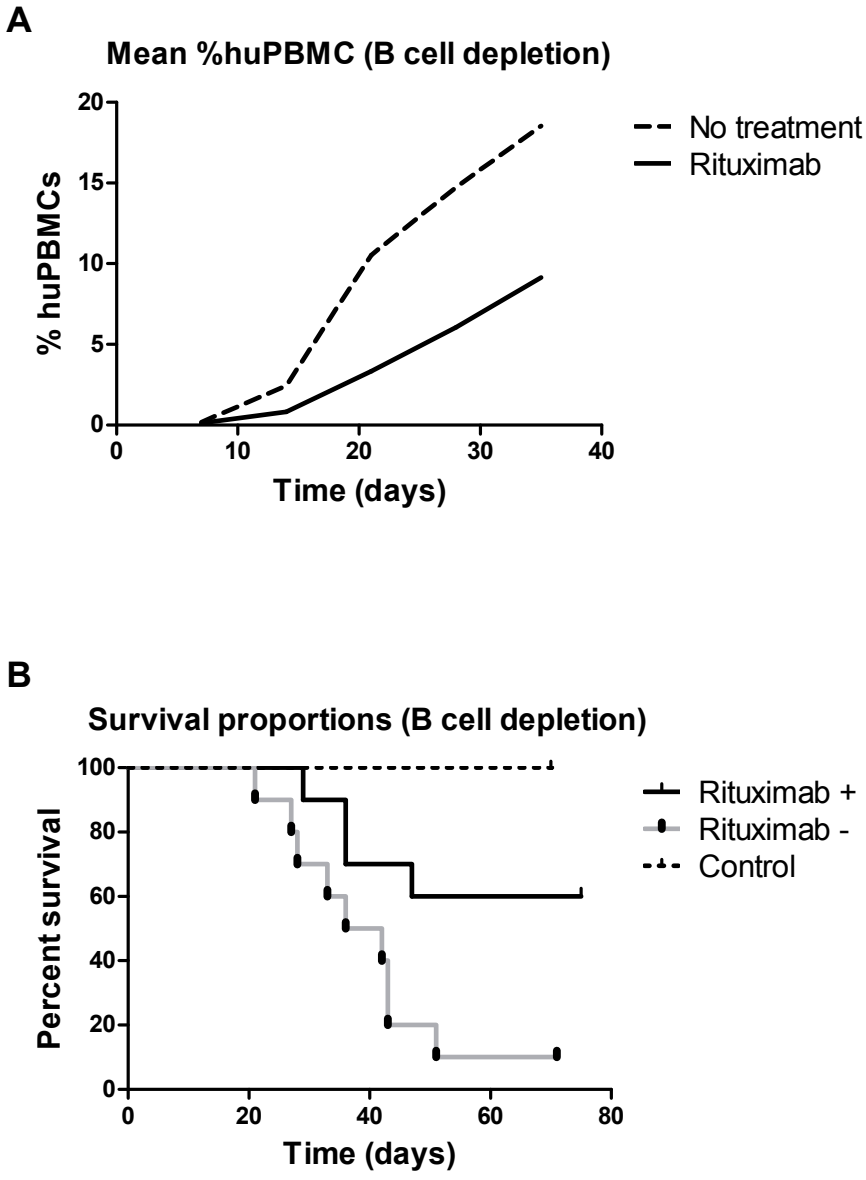

Figure 2. Survival and comparison of fraction reconstituted huPBMC's in B cell depletion. A: The difference in percentage of huPBMC's in mice either treated with Rituximab to induce B cell depletion or not treated with Rituximab, and B: A comparison of the survival portions between the RAG2 $^{-/-}$ γc$^{-/-}$ mice with and without Rituximab treatment versus control mice (n= 10, consisting of 5 RAG2 $^{-/-}$ γc$^{-/-}$ mice with Rituximab but no huPBMC's and 5 RAG2 $^{-/-}$ γc$^{-/-}$ control mice (with no Rituximab nor huPBMC's).

Macrophage depletion did not show any effect on the number of Tregs in the morphological evaluation.

B cell depletion model

Infiltration patterns of cells and fibrosis followed the known pattern for this xenogeneic model from our previous description [28]. Early depletion of B cells showed a significant decrease in the numbers of B cells and plasma cells in spleen and liver (in which the Rituximab treated huPBMC injected group did not show any B cells nor plasma cells at all), as well as a significant decrease of fibrosis in the liver (Table 2). When comparing only the huPBMC injected groups, a significant decrease was shown for the presence of B cells and plasma cells in the lung ($p<0.01$ and $p<0.05$ respectively) in which again no B cells or plasma cells were detected in Rituximab treated huPBMC injected mice, as well as a significant decreased presence of T cells in the gut ($p<0.05$).

Cytokine profile

Macrophage depletion model

The RNA quality from the first experiment was not sufficient for cytokine analysis. Cytokine analysis performed in the second experiment showed that human macrophages (huCD68) were not present (cycle thresholds were above 30 in all samples). No significant difference was detected for murine macrophages (mouse CD68), more specifically no differences were detected in spleen ($p=0.2284$) nor liver ($p=0.1304$). Minor significant differences were detected for other cytokines (Figure 3). Murine IFN-γ was not detected, but murine TGF-β, CTGF and BMP4 were present in several organs as well as human TGF-β and IFN-γ and RNA specific for Tregs (FOXP3), Th1 cells (Tbet) Th2 cells (GATA3) and Th17 cells (RORC).

A decrease for human TGF-β was detected in the lungs and an increase in mouse CTGF in bone marrow. Although human IFN-γ levels in several organs seemed to be lower after macrophage depletion, this effect was not significant. Comparing mRNA levels for specific cells markers (Figure 4) showed that in contrast to the histological analysis, a significant increase in human FoxP3 was found in spleen ($p=0.0038$) as well as in bone marrow ($p <0.001$). In the experiments with macrophage depletion, the levels of FoxP3 positive cells were usually higher in these organs (figure 4).

B cell depletion model

Similar cytokine analysis in our B cell depletion model (figure 5) confirmed the presence of murine macrophages and murine markers CTGF, BMP4 and TGF-β. No Murine IFN-γ was detected. Human markers FG2, PDGF, EGF, BMP4 and CTGF were not detected, but variable RQ values for human TGF-β, IFN-γ, FoxP3, CXCR4, GATA3, Tbet, PAI and RORC were detected.

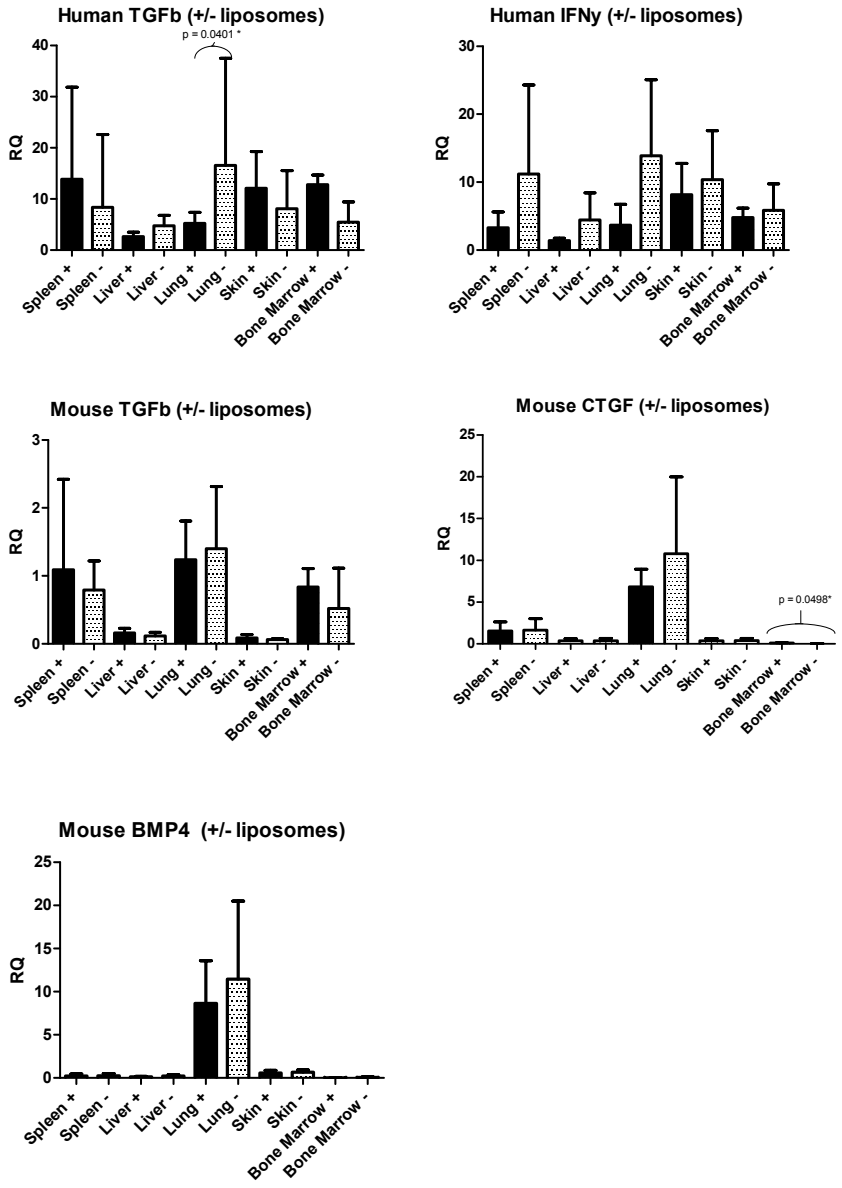

Figure 3. Expression of mRNA for fibrogenic proteins in macrophage depletion. The RQ values of mRNA expression are shown from macrophage depletion experiment 2, for RNA for humanTGF-β, human IFN-γ and for murine proteins TGF-β, CTGF and BMP4 in spleen, liver, lung, skin and bone marrow of RAG2 $^{-/-}$ γc$^{-/-}$ mice injected with huPBMC's with macrophage depletion due to clodronate containing liposomes (indicated as +) and RAG2 $^{-/-}$ γc$^{-/-}$ mice injected with huPBMC's without this depletion (indicated as -). No murine IFN-γ was detected.

Table 1. Statistical analysis of histology and immunohistochemistry data in macrophage depletion.

				Fibrosis			T cells		
			N (+/- liposomes)	effect of liposomes	p value	significant	effect of liposomes	p value	significant
Spleen	Exp. 1	Single donor	10(3/7)	↓	0,22	no	↓	0,22	no
	Exp. 2	Pooled data (donor A+B)	16 (8/8)	↑	0,28	no	↑	0,16	no
		Donor A only	*9 (5/4)*	↓	*0,0035*	*yes*	↓	*0,1131*	*no*
		Donor B only	*7 (3/4)*	↑	*0,5514*	*no*	↑	*0,0321*	*yes*
Liver	Exp. 1	Single donor	10(3/7)	↓	0,06	no	↓	0,11	no
	Exp. 2	Pooled data (donor A+B)	16 (8/8)	↑	0,1	no*	↑	0,0006	yes
		Donor A only	*9 (5/4)*	↑	*0,0031*	*yes**	↑	*0,0001*	*yes*
		Donor B only	*7 (3/4)*	↓	*0,6447*	*no*	↑	*0,3743*	*no*
Lung	Exp. 1	Single donor	10(3/7)	↓	0,21	no	↓	0,31	no
	Exp. 2	Pooled data (donor A+B)	16 (8/8)	↓	0,25	no*	↑	0,01	yes
		Donor A only	*9 (5/4)*	≈	*0,393*	*no**	↑	*0,05*	*yes*
		Donor B only	*7 (3/4)*	↓	*0,5206*	*no**	↑	*0,69*	*no*
Skin	Exp. 1	Single donor	10(3/7)	≈	1	no	↓	0,27	no
	Exp. 2	Pooled data (donor A+B)	16 (8/8)	≈	0,35	no	↑	0,0044	yes
		Donor A only	*9 (5/4)*	↑	*0,23*	*no**	↑	*0,036*	*yes*
		Donor B only	*7 (3/4)*	≈	*0,71*	*no*	↑	*0,1097*	*no*
Colon	Exp. 1	Single donor	10(3/7)		#	#	≈	0,89	no
	Exp. 2	Pooled data (donor A+B)	16 (8/8)	↑	0,792	no*	↑	0,0137	yes
		Donor A only	*9 (5/4)*	≈	*0,9438*	*no**	↑	*0,018*	*yes*
		Donor B only	*7 (3/4)*	↑	*0,7439*	*no**	↑	*0,5864*	*no*
Femur	Exp. 1	Single donor	10(3/7)	↑	0,68	no	↑	0,59	no
	Exp. 2	Pooled data (donor A+B)	16 (8/8)	↑	0,0005	yes	↑	0,0001	yes
		Donor A only	*9 (5/4)*	↑	*0,0075*	*yes*	↑	*0,0012*	*yes*
		Donor B only	*7 (3/4)*	↑	*0,076*	*no*	↑	*0,1251*	*no*

Mean survival with Standard Deviation (SD) an p-values for histological and immunohistochemical scoring in macrophage depletion experiments 1 and 2, each experiment comparing mice injected with huPBMC's with macrophages depletion by clodronate containing liposome-injection (liposomes+) and mice injected with huPMBC's without clodronate containing liposome-injection (liposomes -). The encountered effect related to liposomes injected is indicated with arrows indicating an associated increase or decrease of the mean histological infiltration scores.

B cells (CD79a)			B cells (CD20)			Plasma cells			Tregs		
effect of liposomes	p value	significant	effect of liposomes	p value	significant	effect of liposomes	p value	significant	effect of liposomes	p value	significant
↓	0,11	no	↑	0,11	no	↓	0,68	no	↑	0,21	no
↑	<0.0001	yes	↑	<0.0001	yes	↑	0,0008	yes		#	#
↑	*<0.0001*	*yes*	↑	*0,0314*	*yes*	↑	*<0.0001*	*yes*		#	#
↑	*0,0013*	*yes*	↑	*0,5857*	*no*	↑	*0,2112*	*no*		#	#
↓	0,04	yes	↑	0,01	yes	↓	0,48	no	↑	0,17	no
↑	0,002	yes	↑	0,002	yes	↑	0,0003	yes		#	#
↑	*0,0005*	*yes*	↑	*0,0431*	*yes*	↑	*0,006*	*yes*		#	#
↓	*0,4178*	*no*	↑	*0,2367*	*no*	↑	*0,0101*	*yes*		#	#
↓	0,06	no	↑	0,04	yes	↓	0,06	no	↑	0,1	no
↑	0,028	yes	↑	0,028	yes	↑	0,0004	yes		#	#
↑	*0,4231*	*no*	↑	*0,1094*	*no*	↑	*0,0202*	*yes*		#	#
↑	*0,0033*	*no*	↑	*0,8255*	*no*	↑	*0,0007*	*yes*		#	#
↓	0.11**	no**		#	#	↓	0,22	no	≈	1,00	no
↑	0,0237	yes*	↑	0,0237	yes*	↑	0,548	no		#	#
↑	*0,013*	*yes**		*#*	*#*	↓	*0,3927*	*no*		#	#
	#	*#*		*#*	*#*		*#*	*#*		#	#
	#	#		#	#		#	#	↑	0,72	no
↑	0,88	no		#	#	≈	0,486	no		#	#
↑	*0,5669*	*no*		*#*	*#*	↑	*0,3342*	*no*		#	#
↓	*0,4178*	*no*		*#*	*#*	↓	*0,4178*	*no*		#	#
↑	0,89	no	↑	0,01	yes	↑	0,04	yes	↑	0,46	no
↑	0,0534	no	↑	0,0534	no	↑	0,253	no*		#	#
↑	*0,0057*	*yes*	↑	*0,1074*	*no*	↑	*0,553*	*no**		#	#
↑	*1,00*	*no*	↑	*!*	*!*	↑	*0,3165*	*no*		#	#

no cells/ fibrosis detected

* significant difference between scoring of the analysts

** morphologically plasma cells with CD79a staining

! number of available data not sufficient for analysis

↑ mean score liposomes > mean score without liposomes

↓ mean score liposomes < mean score without liposomes

≈ mean scores with and without liposomes almost equal

Table 2. Statistical analysis of histology and immunohistochemistry data in B cell depletion.

	Fibrosis			T cells		
	effect of Rituximab	p value	significant	effect of Rituximab	p value	significant
Spleen	↑	0,15	no	≈	0,92	no
Liver	↓	0,05	yes	↓	0,20	no
Lung	↓	0,82	no	≈	1,00	no

Indicating the observed effect (increase or decrease of the encountered cell numbers) associated with the use of Rituximab in the huPBMC injected groups, with the P-values for histological and immunohistochemical scoring in mice injected with huPBMC's with and without Rituximab treatment and the control group without huPBMC injection.

B cells			Plasma cells			Tregs		
effect of Rituximab	p value	significant	effect of Rituximab	p value	significant	effect of Rituximab	p value	significant
↓	0,03	yes	↓	0,03	yes	↓	0,80	no
↓	0,03	yes	↓	0,05	yes	≈	0,92	no
↓	0.08*	no*	↓	0.14**	no**	↓	0,45	no

* overall effect, effect in huPBMC injected groups significant ($p < 0.01$) lacking B cells in the huPBMC injected group treated with Rituximab.

** overall effect, effect in huPBMC injected group significant ($p < 0.05$) lacking plasma cells in the huPBMC injected group treated with Rituximab

↑ mean score human cells Rituximab treated group > mean score human cells without Rituximab treatment

↓ mean score human cells Rituximab treated group < mean score human cells without Rituximab treatment

≈ mean score human cells Rituximab treated and non-treated group almost equal

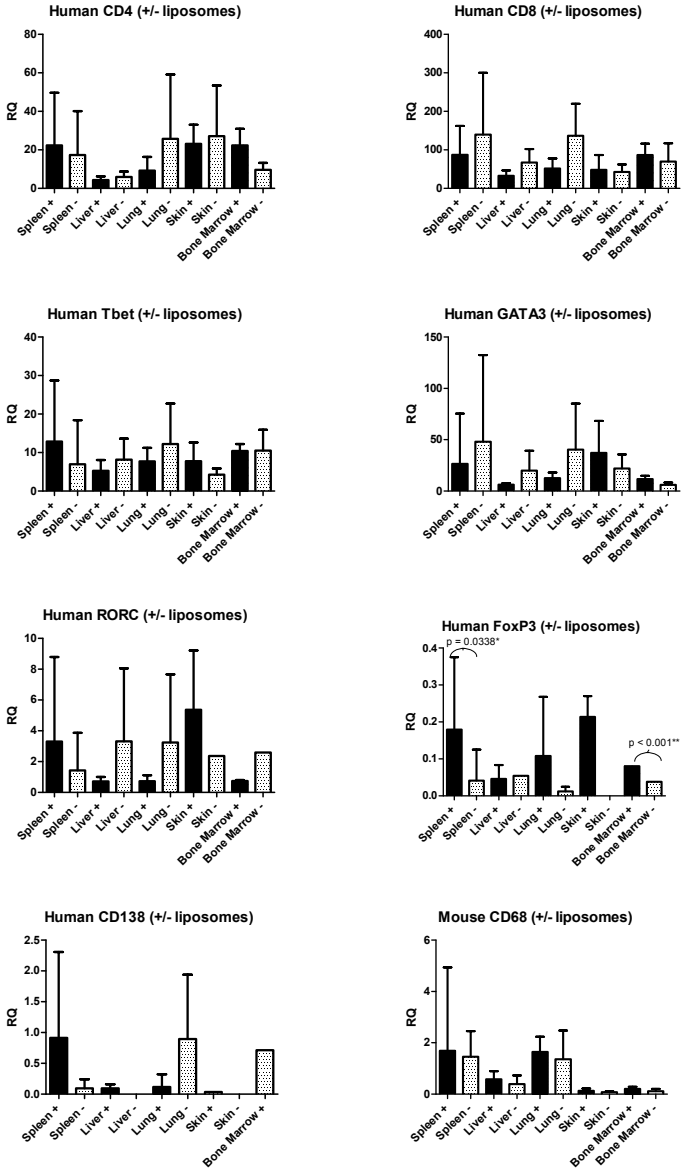

Figure 4. Expression of mRNA for specific cell marker mRNA in macrophage depletion. RQ values are shown from macrophage depletion experiment 2 (RAG2 $^{-/-}$ γc$^{-/-}$ mice injected with huPBMC's with macrophage depletion due to clodronate containing liposomes (indicated as +) and RAG2 $^{-/-}$ γc$^{-/-}$ mice injected with huPBMC's without this depletion (indicated as -), for different human cell types, including FoxP3 (Tregs), GATA3 (Th2 cells), Tbet (Th1 cells), RORC (Th17 cells), CD4 (T helper cells), CD8 (cytotoxic T cells) and CD138 (plasma cells) as well as for murine CD68 (murine macrophages). Human CD68 showed cycle threshold values above 30 only and human macrophages were therefore considered as virtually not present.

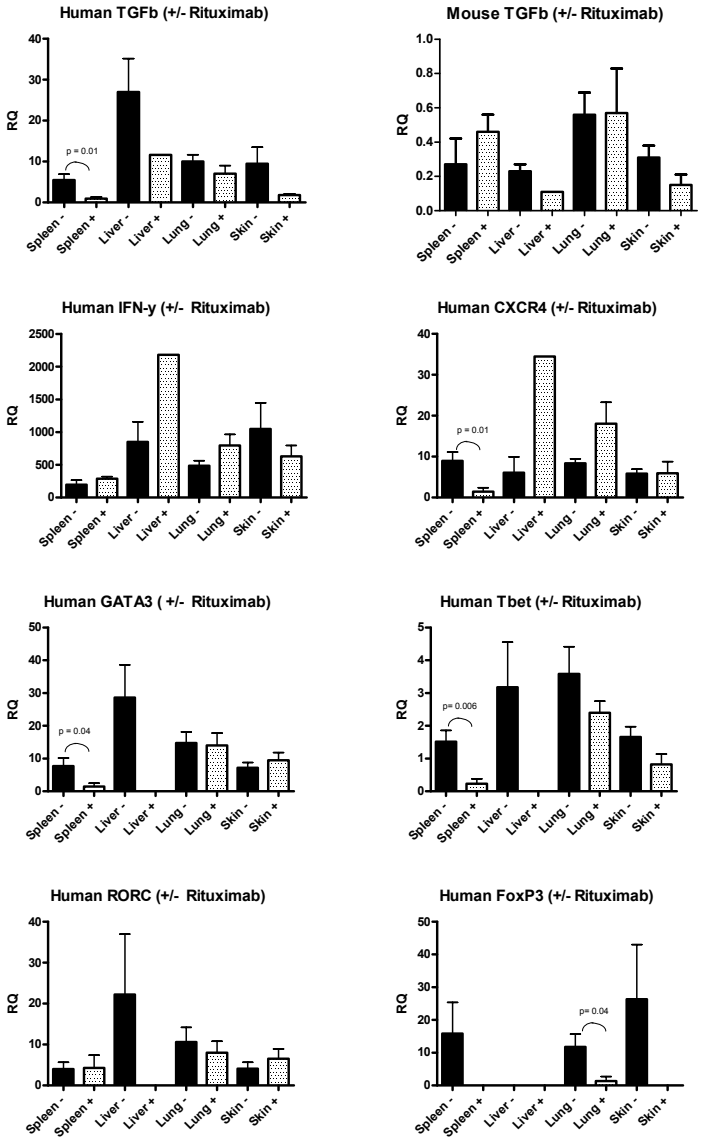

Figure 5. Expression of mRNA for fibrogenic proteins and specific cell marker mRNA in early B cell depletion. RQ values are shown from the early B cell depletion experiment (RAG2 $^{-/-}$ γc$^{-/-}$ mice injected with huPBMC's with Rituximab induced B cell depletion (indicated as +) and RAG2 $^{-/-}$ γc$^{-/-}$ mice injected with huPBMC's without this depletion (indicated as -), for the most important observations, concerning human and murine TGF-β, human IFN-γ and different human cell types, including FoxP3 (Tregs), GATA3 (Th2 cells), Tbet (Th1 cells). All other results were either not significant (CD4, CD8, murine CD68, murine CTGF, murine BMP4), had cycle threshold (Ct) values > 35 (CD20 and human CD68) or were not detectable at all (human factors FGF2, PDGF, EGF, BMP4 and CTGF).

The results showed a significant difference for human TGF-β in the spleen (p = 0.01), levels being higher in mice with Rituximab treatment. Human TGF-β levels in liver also seem to be higher, but as only one mouse could be evaluated with Rituximab treatment, the significance of this difference could not be established. A similar problem occurred for FoxP3, CXCR4, GATA3, Tbet and RORC (number of adequate samples for evaluation were too low), although spleen and lung both show a significant higher level of FoxP3 in mice without treatment (p= 0.04) and CXCR4, GATA3 and Tbet also were significantly increased in spleen (p=0.01, p=0.04 and p=0.006) in the non-treated group. We did not find significant differences in the number of CD4+ or CD8+ cells.

DISCUSSION

Our study evaluated the effect on survival, histomorphological changes and cytokine assays in macrophage depletion as well as early B cell depletion in a xenogeneic mouse model for GvHD. Although our experiments may show a substantial difference in number of and gender mice used (as the number of mice treated was subject to availability of cells and clodronate as well as the availability of an adequate number of mice of similar gender and age), we consider the results of our experiments sufficient to address the issue of possible significant effects of modifications in xenogeneic mouse models for GvHD when using macrophage- or B cell depletion protocols, even when only single dose treatment of either clodronate containing liposomes of Rituximab has been used.

With depletion of macrophages, survival was significantly impaired. Histologically, the number of B cells and occasionally plasma cells in liver, lung and femur were altered. The effect on histological presence of fibrosis was unremarkable and only visible in femur. The molecular analysis showed a decrease in human TGF-β in lungs and an increase in murine CTGF in bone marrow after our clodronate depletion protocol. We did not find significant changes in murine CD68 in our qPCR analysis, though we believe that this reflects the known regeneration of murine macrophages after a single dose treatment with clodronate containing liposomes as reported in previous literature [22, 24, 25] as our qPCR analysis was performed at least 13 days post-treatment and regeneration starts within 5 days in liver and within a week in spleen.

With our early B cell depletion experiment, the clinical effect was precisely opposite to macrophage depletion. Survival was clearly improved, even though the number of circulating huPBMC's did not change significantly. In addition to histological confirmation of B cell depletion, the histomorphological analysis only showed a change in liver fibrosis and no significant alterations in infiltrating T cells or Tregs. The cytokine analysis in our Rituximab experiment showed several interesting observations.

There were decreased human TGF-β levels in spleen, as well as decreased levels for CXCR4, GATA3 (Th1 cells) and Tbet (Th2 cells). The observed reduction in CXCR4 in spleen suggests that lymphocytes are less drawn to the spleen after early Rituximab treatment. Our results may suggest an increase in CXCR4 in liver, lungs and skin, indicating that perhaps these organs become more attractive to lymphocytes after Rituximab treatment. However, the qPCR results in these organs were not significant. The decrease in GATA3 and Tbet without changes in the number of CD4+ and CD8+ cells (neither histologically nor in qPCR) remains difficult to explain. One might expect an increase in Th17 cells (with an increase in RORC in qPCR analysis) to compensate and explain our histological data, but our qPCR data could not confirm this (no significant changes in RORC were seen). It will be interesting to look into this in more detail in future experiments.
Our clinical macrophage depletion results are in line with the clinical effect as described in literature, including the lack of human macrophages in general after introducing human cell-mixtures [26, 29]. The obtained reaction develops faster and symptoms are more severe after macrophage depletion. Our results show however, that the number of infiltrating B cells and occasionally plasma cells might significantly increase after a macrophage-depletion protocol. To our knowledge, this is a new observation that has not been described before.

Our early B cell depletion experiment also follows the known pattern of affected survival, but despite the clear clinical effect, the morphology in the evaluated murine organs only showed a difference in liver fibrosis between mice treated and not treated with Rituximab.

The observation of only alterations in liver fibrosis and the effect on TGF-β does not contradict the encountered profound clinical effect. Liver fibrosis causes fatigue, nausea and weight loss and in our model, we can only conclude that the liver fibrosis is due to a xenogeneic GvHD.

However, as we could only establish a difference in liver disease, the clinical improvement therefore does not imply that the effects account for all clinical features of GvHD (like e.g. cutaneous involvement). Also, in human other therapeutic agents or co-morbidities may have an effect on liver function as well and with that become a confounder in the evaluation of e.g. Rituximab on clinical performance. The increase in human TGF-β is in line with previous literature [6], and might be the cause for our liver fibrosis as murine CTGF and murine BMP4 did not show significant changes. TGF-β is known for its effects on inducing injury in hepatocytes [30, 31]. The levels of TGF-β in the liver could unfortunately not be evaluated for significance, as only a small number of mice treated with Rituximab had an adequate RNA sample to process. *If* this would follow the significance as seen in spleen and lungs for this profibrogenic protein, this could be an explanation for the cause of the fibrosis.

Our Q-PCR data also suggest an interplay between human TGF-β and murine tissue. This is in line with the suggestion by MacDonald K.P.A *et al* [32], that TGF-β is indeed derived from engrafting donor-cells, although they correlated this to donor macrophages and we only affected donor B cells in this experiment. For donor macrophages all cycle thresholds in our Q-PCR analysis were above 30, concluding that virtually no donor macrophages were present. Only host macrophages could be detected. It is known that host macrophages (in their function as antigen presenting cells) are related to the occurrence of GvHD [33]. Our macrophage-depletion experiments confirm the importance of host macrophages in GvHD. The observed reduced survival due to depletion of host macrophages in our experiments could possibly be related to the loss of Toll-like receptor 4 (TLR-4) in macrophages, as described by Imado *et al* [34]. However, further research is suggested to unravel the possible interactivity between host, donor macrophages (if present) and (perhaps different activation-stages of) donor B cells in GvHD.

It would be very interesting to correlate our histological findings with clinical hepatic GvHD. So far, no literature was found on the effects of Rituximab in hepatic GvHD specifically. Nevertheless, for cutaneous GvHD a correlation between TGF-β and the length of survival has already been shown, even though the results show that in humans the relation probably only accounts for acute GvHD and lichenoid chronic GvHD [35]. It supports our findings of the importance of TGF-β in GvHD, although our study is showing a more sclerotic type of response. With regard to Tregs, our B cell depletion experiment showed two interesting features.

First, in our mice with less symptoms (after Rituximab treatment) mRNA levels of FoxP3 were significantly lower compared to those with a more profound reaction, although literature suggests that higher levels of Tregs should correlate with increased survival. However, the low mRNA level of FoxP3 was not accompanied by a change in Treg infiltration histologically, suggesting that the mRNA level of FoxP3 is not always linear related to the actual number of Tregs. Either it reflects the difficulty of scoring less-frequent cells histologically, or the FoxP3 protein might be upregulated within single Tregs without a significant increase in the number of Tegs. Our observation is consistent though, with the findings of WU *et al* [35] in their skin biopsies.

Second, when correlating our results with literature the findings of lower mRNA levels for FoxP3 in spleen and lung after Rituximab treatment suggests that our early B cell depletion affected mostly inactivated B cells [11]. Indeed, we introduced Rituximab almost immediately after the introduction of huPBMC's. We did not know the number of activated B cells in our huPBMC mixture, nor the number of CD20+CD5+ cells (an indicator for possible clinical response to treatment [7]) and therefore these findings might well be related to each other.

In summary, we confirm known clinical effects of depleting macrophages or B cells in a GvHD mouse model, but our experiments showed that histological and cytokine assay results can be significantly influenced by depletion of either of these cell lines as well. For both cell lines the effect of depletion on histological changes can be unexpected, despite significant effects on survival. Cytokine-analysis might however help explain some of the encountered contradictions and the interplay between donor and host cells. We therefore, strongly continue to recommend researchers interested in GvHD to always perform a full triad analysis of clinical symptoms, histology as well as cytokine profile (specified not only for peripheral blood, but also other involved organs in the reaction).

DECLARATIONS

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Data availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest disclosure

The authors have no conflicts of interests to disclose.

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SUPPLEMENT SECTION

Appendix A. Antibodies used for immunohistochemical detection of human and murine cells after huPMBC injection in RAG2-/-γc-/- mice.

Primary Antibody	Manufacturer	Animal	Monoclonal or Polyclonal
Hu-CD2	Novocastra	Mouse	Monoclonal
Hu-CD4	Monosan Xtra	Mouse	Monoclonal
Hu-CD8	DakoCytomation	Mouse	Monoclonal
Hu-CD20	DakoCytomation	Mouse	Monoclonal
Hu-CD79a	DakoCytomation	Mouse	Monoclonal
Hu-CD138	Serotec	Mouse	Monoclonal
Hu-κ	DakoCytomation	Rabbit	Polyclonal
Hu-λ	DakoCytomation	Rabbit	Polyclonal
Hu-plasmacell	DakoCytomation	Mouse	Monoclonal
Hu-CD68	Novocastra	Mouse	Monoclonal
Hu-FoxP3	Ebioscience	Rat	Monoclonal
Mouse-CD68	Hycult Biotech	Rat	Monoclonal

Appendix B. Abbreviations used in qPCR analysis

Abbreviations used in qPCR analysis regarding specific cytokine/protein/gene function	
TGF-β / TGFb	Transforming growth factor beta
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IFN-γ	Interferon gamma
CXCR4	Chemokine receptor type 4
FGF2	Fibroblast growth factor 2
PDGF	Platelet derived growth factor
EGF	Epidermal growth factor
BMP4	Bone morphogenetic protein 4
CTGF	Connective tissue growth factor
Abbreviations used in qPCR analysis regarding specific cell types	
FoxP3	Abbreviation for Forkhead protein 3, used as signature for regulatory T cells (Tregs)
GATA3	Transcription factor involved in T -helper 2 (Th2) cell differentiation
Tbet	Transcription factor associated with T-helper 1 (Th1) cell differentiation
RORC	Signature for T-helper 17 (Th17) cells
CD4	T-helper cell cluster
CD8	Cytotoxic T cell population
CD20	B cell population
CD68	Macrophage population

Clone	Lot	Dilution	Pretreatment
AB75	127119	1:160	EDTA
4B12	247919	1:200	EDTA
C8/144B	51182	1:200	Citrate
L26	83	1:400	Citrate
JCB117	42791	1:200	Citrate
B-B4	605	1:1000	Citrate
-	44399	1:10000	Citrate
-	120	1:20000	Citrate
VS38C	38925	1:400	EDTA
KP1	211708	1:800	Citrate
PCH101	E021753	1:200	Citrate
FA-11	4817M21	1:50	None

Multifunctional cytokine known for its role in regulating inflammatory processes and involved in proliferation, activation and chemotaxis of many immune cells.
Protein receptor involved in downregulation of immune responses, expressed on T cells
Protein coding gene, known as 'housekeeping' gene.
Important regulating cytokine in inflammatory processes, with immunostimulatory and -modulating effects.
Receptor for stromal derived factor 1 (chemotactic for lymphocytes and important for hematopoetic homing of stem cells)
Fibrosis related factor with mitogenic effect on fibroblasts
Fibrosis related factor with mitogenic effect on fibroblasts
Promotor of TGF-β related fibrosis
Proinflammatory gene with profibrotic potential
Profibrotic cytokine

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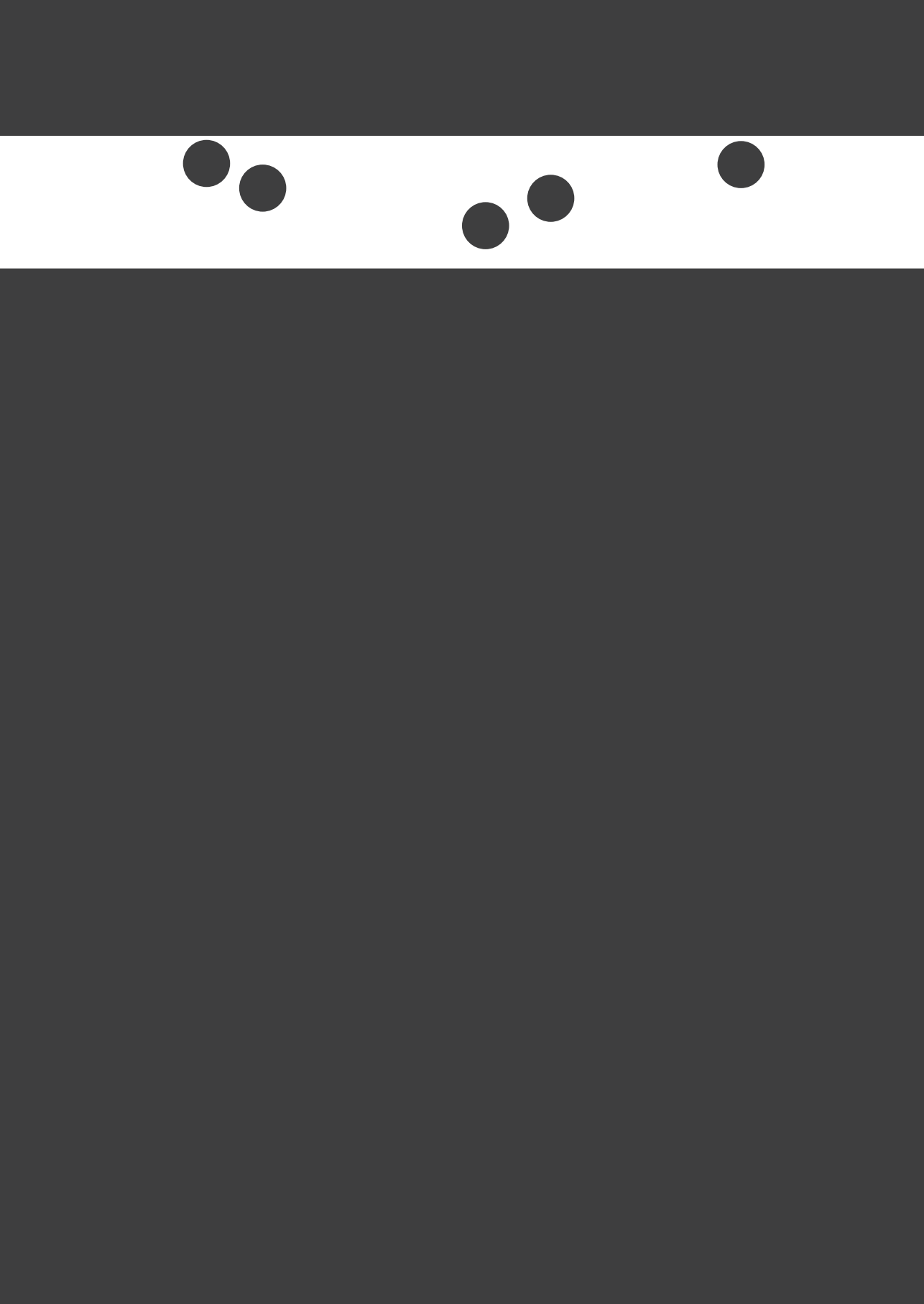

CHAPTER 5

SUSCEPTIBILITY OF REGULATORY T CELLS IN VITRO AND IN VIVO TO B CELL ACTIVATION AND MYELOMA IN A GRAFT-VERSUS-HOST RESPONSE

Marieke C.H. Hogenes[1,2], Teun Guichelaar[3], Natalie ter Hoeve[2], Tuna Mutis[4], Roel A. de Weger[2]

[1]Laboratory for Pathology East Netherlands
[2]Department of Pathology UMC Utrecht,
[3]National Institute for Public Health and the Environment (RIVM), Bilthoven,
[4]Department of Haematology VU University Medical Centre Amsterdam

Submitted

ABSTRACT

A graft-versus-host response (GvHR) after bone marrow transplantation can be life-threatening. Many researchers currently focus on the role of B cells and regulatory T cells (Tregs) in this response for improvement of treatment options. This paper evaluates interaction and collaboration between (in)activated B cells and Tregs in this GvHR.

An *in vitro* study was done to evaluate the different stages of activation of B cells.

An *in vivo* study was done to compare the *in vivo* effect on GvHR histology and clinical symptoms of GvHR with myeloma as a plasma cell in combination with huPBMCs with and without additional Tregs in $RAG2^{-/-}\gamma c^{-/-}$ mice.

Results showed that B cell activation significantly boosted T cell proliferation in the GvHR, but also boosted the development of Tregs. Interaction between Tregs and B cells was confirmed in *in vitro* assays. *In vivo* the injection of huPBMC resulted in a GvHR. Although myeloma activity declined and peripheral blood counts of T cells significantly lowered after addition of extra Tregs, survival did not significantly improve when adding extra Tregs. Histologically the addition of Tregs did not significantly alter the GvHR for fibrosis development and the myeloma presence and infiltrate analysis showed only minor changes.

Our results suggest that in order to reduce GvHR, early B cell depletion might be preferred. Activated B cells and myeloma boost Treg proliferation within a GvHR, but the functional Treg activity in the presence of these activated B cells is not sufficient to reduce GvHR within affected organs.

INTRODUCTION

Graft-versus-host disease (GvHD) is a challenging aspect in patients receiving stem cell transplantation during treatment of e.g. haematological malignancies. Morbidity and mortality of GvHD remains a major concern and prevention therefore, needs further optimisation in treatment protocols [1, 2].

The past few years attention has been drawn to the depletion of B cells with Rituximab in an attempt to reduce the pathogenic graft-versus-host response (GvHR) [3-6]. Unfortunately, not all patients appear to benefit from Rituximab. This might be related to imbalances in B cell homeostasis [7, 8], B cell activation status of donor cells [6, 9], the specific role of Syk signalling in allogeneic B cells [10] and cytokine B cell activating factor (BAFF) [11].

Furthermore, an important role for regulatory T cells (Tregs) in GvHD has been suggested. A high quantity of Tregs is associated with less intense GvHD, though this might be influenced by inflammatory cytokines like IFN-γ [12, 13]. Tregs are a subset of cells with suppressive function on alloreactive T cells and are differentiated in 3 groups: thymus-derived Tregs, peripheral derived Tregs and *in vitro* induced Tregs [14]. However, when research is done with Tregs, they are usually just referred to as a $CD4^+CD25^+FOXP3^+$ T cell population.

Whether B cell activation status correlates or interacts with the quantity and/or function of Tregs is an interesting question, but unknown.

In previous research early B cell depletion showed a prominent increase in mRNA levels characteristic for Tregs [15]. Other research suggest that inactive B cells expand Tregs, either by prolonging life-span or by means of active expansion due to TGF-β production [16]. Another hypothesis is, that Tregs directly supress B cells in the follicular mantle zone [17].

In patients with plasma cell disorders like multiple myeloma and monoclonal gammopathy of undetermined significance (MGUS), the natural number of Tregs is increased [18, 19] although they might be dysfunctional. Regarding myeloma-cells in their nature of being plasmacytoid cells and therefore, derived from the activated B cell lineage, this implies that activation might be related to higher Tregs levels. Nevertheless, the FoxP3 expression is usual lower in myeloma and MGUS cases and might affect suppressive activity of Tregs, even when extra Tregs are added [18, 19].

To evaluate whether intervention at specific activation stages of B cells might stop the progression of a GvHR, we studied the importance of B cell activation with respect to its influence and interaction with Tregs in a GvHR in a combined *in vitro* and *in vivo* study.

MATERIALS AND METHODS

Isolation of human blood cells and activation of B cells

Human peripheral blood mononuclear cells (huPBMCs) were isolated from healthy donor volunteers (Sanquin Blood Bank, Amsterdam, The Netherlands) by Ficoll Isopaque density gradient centrifugation following known protocols [20, 21].

Human inactive B cells ($CD19^+CD25^-$), responder T cells ($CD4^+CD25^-$, abbreviated as Tresp) and regulatory T cells ($CD4^+CD25^+$, abbreviated as Tregs) were isolated from the huPBMCs using immunomagnetic isolation beads. First, the huPMBC population was depleted for the $CD25^+$-fraction, using CD25 MicroBeads II (Miltenyi Biotec, Bergisch Gladbach, Germany). B cells were then purified from the $CD25^-$-fraction by means of the MiniMACS separation method (Miltenyi Biotec) using anti-CD19-PercP antibody (BD-Pharmingen, San Jose, US) and Tresp cells were isolated similarly from the CD25-depleted fraction by using anti-CD4 antibody (BD Pharmingen). Tregs were isolated from huPBMCs using a $CD4^+CD25^+$ Regulatory T cell isolating kit (Miltenyi Biotec), according to the manufacturer's instructions.

B cell activation was performed using an irradiated 293 CD40L-celline (kindly provided by Dr. A.C.M. Martens, Department of Haematology, VU University Centre Amsterdam, The Netherlands) in RPMI-1640 medium (ThermoFisher Scientific, Paisly, Scotland) with 10% human AB serum (Sanquin, Amsterdam Netherlands) and 100 U/ml Penicillin Streptomycin Solution (ThermoFisher Scientific) in co-culture with previously isolated inactive B cells. B cell activation culture was performed for 3-5 days at 37°C and 5-6% CO_2. Activated $CD19^+CD25^+$ B cells were then isolated using the MiniMACS separation method with CD19-antibody and the success of the B cell activation culture was determined by FACS analysis using CD19-APC (BD Biosciences), CD25-PE (Milteny Biotec) and CD86-FITC (BD-Pharmingen).

Luciferase-labelling of myeloma cell lines U266 and RPMI-8226 for use *in vivo* was carried out as described by Rozemuller *et al* [21] and clodronate containing liposomes for macrophage depletion were prepared as described by van Rijn *et al* [20].

Ex vivo expansion of the isolated Tregs was performed prior to the *in vivo* study, using anti-CD3/anti-CD28 expander beads (Invitrogen Dynal AS, Oslo, Norway) as described previously [22].

In vitro assay

In vitro assays for the assessment of Treg function in relation to B cell activation status in presence and absence of stroma cells (stromal cell-line HS-5 ATCC) were performed in quadruplicate. In summary a Tresp proliferation was induced *in vitro* and their proliferation was compared to Tresp proliferation in the presence of either resting B cells, activated B cells or multiple myeloma cells, in the presence or absence of Tregs. A similar evaluation was performed in the presence of stroma cells to enable a better comparison of the results of the *in vivo* study.

For each in vitro assay, CD4+CD25+ Tresp were CFSE labelled and CD4+CD25+ Tregs PKH labelled (both Sigma-Aldrich, Steinhem, Germany) prior to culture. (In)activated B cells, U266 myeloma-cells, Tregs and Tresp were cultured on a 96-well plate (Costar, Sigma Aldrich, Darmstadt, Germany) with anti-CD3/anti-CD28 expander beads (Invitrogen Dynal AS) in RMPI-1640 medium with 10% human AB serum and 100 U/ml Penicillin Streptomycin Solution for 3 days, at 37°C. Concentrations used were $2x10^5$ cells/ml for Tresp and Tregs, $4x10^5$ cells/ml for inactivated, activated B cells as well as U266 myeloma cells and $1x10^5$ cells/ ml for stroma cells, using 50 ml of each cell line per well when appropriate. Validation of the activation status of B cells in this culture was repeated prior to *in vitro* culture by FACS analysis using CD86-PE, CD25-PercP and CD19-FITC as stated above.

Mice and conditioning regiments

The *in vivo* study consisted of two experiments, each using either luciferase-labelled U266 multiple myeloma cell-line or luciferase-labelled RPMI-8226 multiple myeloma cell-line in $Rag2^{-/-}\gamma c^{-/-}$ mice (n=12 and n=14 respectively). The experiments were conducted with permission of the local Ethical Committee for Animal Experimentation in accordance with Dutch national Law.

The methods have been described in detail previously [21-23]. In brief, mice were irradiated and injected with luciferase-labelled multiple myeloma intravenously ($5x10^6$cells / mouse). At day 4, mice were injected intravenously in the tail vein with clodronate containing liposomes and 3 groups were formed. When in the appropriate group, mice received either no additional cells (control group, group A) or huPBMCs containing $1x10^7$ $CD3^+$ T cells (group B) or huPBMCs containing $1x10^7$ $CD3^+$ T cells plus $1x\ 10^7$ expanded $CD4^+CD25^+$ Tregs (group C) intravenously at day 5. Clinical monitoring of mice was carried out similar to previous studies [20, 24] and bioluminescence imaging was performed daily to monitor progression of multiple myeloma load, similar as described by Spaapen *et al* [23].

Mice were sacrificed when weight loss exceeded 20% or more of the original body weight. Peripheral blood was sampled and FACS analysis for T cells with anti-huCD3-FITC (BD Biosciences) was performed, as an indication of T cell proliferation in peripheral blood. Autopsy was performed on all mice to evaluate individual organs.

Histology

At each autopsy, samples were taken from spleen, lung, liver, skin, femur and spine. Samples were formalin fixed and processed for histology using H&E staining as well as immunohistochemistry according to our previously described method for histological analysis [24] for assessment of fibrosis and infiltrate analysis, using CD20 to identify B cells, CD138 for plasma cells and FoxP3 for Tregs.

Statistical analysis

Survival analysis, Log-rank tests and Kruskal-Wallis tests with one-way ANOVA were executed to evaluate all results, using Graphpad Prism (version 5.0). Values of $p<0.05$ were regarded as significant.

RESULTS

Our study used a combination of *in vitro* study and in *vivo* study, to facilitate evaluation of inactive B cells, active B cells and plasmacytoid cells individually. The *in vivo* study analysed a GvHR in presence of plasmacytoid cells and was regarded as the *in vivo* equivalent of the T-responder reaction in response to myeloma and stroma in our *in vitro* study.

In vitro assay

Results of the *in vitro* assays to compare the Tresp proliferation and Treg proliferation in the absence of B cells or the presence of either resting B cells, activated B-cells and myeloma cells, with and without stroma cells are shown in Table 1 A and B, with an overview of the donors used in panel C. Also, a graphic of the Tresp proliferation in assay 1 is shown in Figure 1. All assays showed a significant increase of Tresp proliferation in the presence of activated B cells compared to inactive B cells. In all experiments our data indicated an inhibiting effect of additional Tregs on Tresp proliferation, of which half of the cases showed significance. Moreover, half of the cases confirmed a significant interaction between B cells and Tregs on Tresp proliferation, in which all values of Treg presence were affected by the B cells. In all assays, the presence of Tresp cells as well as activated B cells significantly affected Treg-proliferation, showing less Treg proliferation when no Tresp were present, though more in the presence of activated B cells. Only one assay showed a significant interaction between B cells and Tresponder cells on Treg-proliferation, in which lack of Tresponder cells resulted in only low Treg proliferation in the presence of inactive B cells.

Table 1. The effect of Tregs and B cells on Tresp proliferation in vitro.

A. Tresponder proliferation

	Exp.	Effect on Tresp proliferation	P value	Significant
Tregs presence	1	↓	0.002	*
	2	↓	0.0135	*
	3	↓	0.0834	
	4	↓	0.0518	
B cell activation	1	↑	< 0.0001	*
	2	↑	< 0.0001	*
	3	↑	< 0.0001	*
	4	↑	0.0002	*
Interaction B cells / Tregs	1		0.0007	*
	2		0.1313	
	3		< 0.0001	*
	4		0.7588	

B. Treg proliferation

	Exp.	Effect on Treg proliferation	P value	Significant
Tresponder presence	1	↑	0.0296	*
	2	↑	0.0021	*
	3	↑	0.133	
	4	↑	0.002	*
B cell activation	1	↑	0.0028	*
	2	↑	< 0.0001	*
	3	↑	< 0.0001	*
	4	↑	< 0.0001	*
Interaction B cells / Tregs	1		0.0968	
	2		< 0.0001	*
	3		0.0636	
	4		0.5241	

C. Overview donors used

Exp.	donor T cells	donor Tregs	donor B cells
1	A	A	D
2	B	B	E
3	A	C	F
4	A	A	G

A. The effect of Tregs presence, B cell activation and interaction between Tregs and B cells on proliferation of Tresp cells, indicating a significant decrease of Tresp proliferation in the presence of Tregs and a significant increase in the presence of activated B cells and myeloma, in B. results of Tresp presence and B cell activation on Tregs proliferation, indicating that Tresp presence and activated B cells and myeloma are associated with an increase in Treg proliferation and in C. an overview of donors used in each assay. In all panels significant results are marked with an asterix (*). A graphic example is shown for the Tresp proliferation results from assay 1 in figure 1.

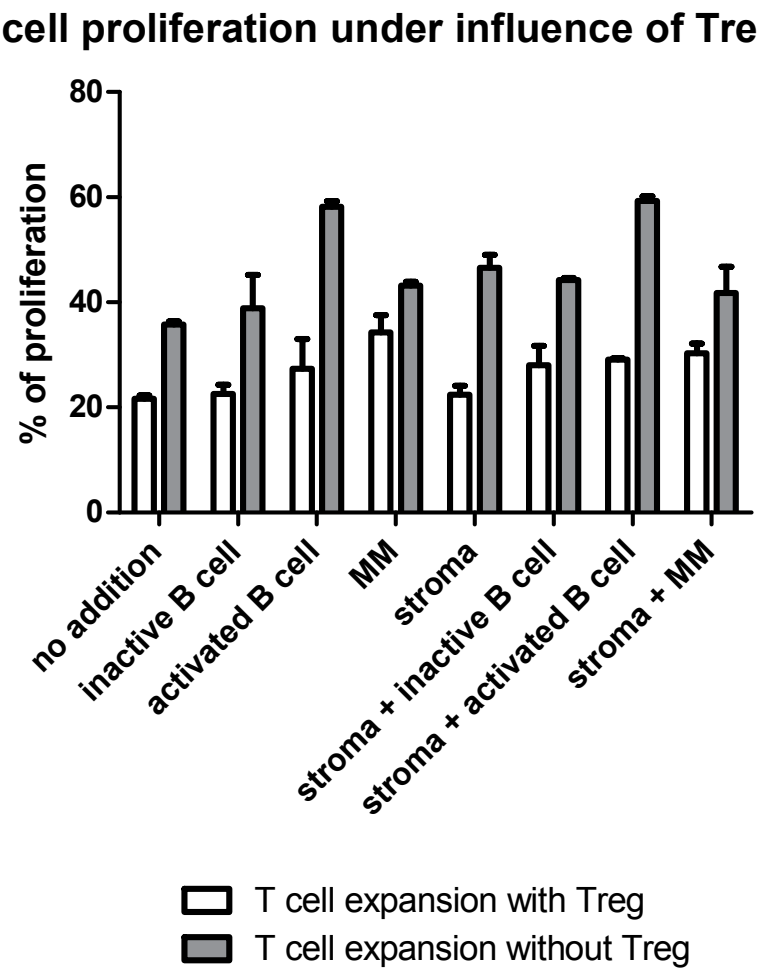

Figure 1. Graphic result of in vitro assay 1, showing the difference in Tresp proliferation in the presence and absence of additional Tregs, when no B cells are present and when either resting B cells, activated -cells of multiple myeloma are present in a situation either with accompanying stroma cells or without stroma cells. There is a significant difference between the groups with and without Treg presence ($p = 0.002$) and a significant difference between the groups comparing the type of B cell present ($p < 0.0001$). However, in this assay there was a significant interaction between B cells and Tregs ($p = 0.0007$), in which Tregs proliferation was also induced by the activation status of the type of B cell present ($p = 0.0028$).

***In vivo* study**

Kaplan-Meier survival curves were plotted for both *in vivo* experiments in which mice were injected with a myeloma cells , myeloma cells combined with huPBMC's, or myeloma cells with huPBMC's enriched with additional Tregs (Figure 2A & 2B), using either U266 (experiment 1) or RPMI-8226 (experiment 2) as myeloma cells.

Neither experiment showed a significant difference in survival when comparing groups with and without additional Tregs (U266 p= 0.5578 and RPMI-8226 p= 0.4189). In peripheral blood a significant reduction of the CD3+-cell count was seen in the presence of additional Tregs ($p<0.05$) (Figure 2C). Luciferase activity showed growth of U266 as well as RMPI-8226 cells after myeloma-inoculation, but a visual decrease in luciferase-activity several days after mice were injected with huPBMCs shows a graft-versus-myeloma response (Figure 2D & 2E).

At autopsy, the presence of myeloma and analysis of the histological GvHR was evaluated with light microscopy. Histomorphology (Table 2) showed a (sclerotic) GvHR in all mice injected with huPBMC (either with or without additional Tregs), with a difference in the extend of the cutaneous fibrosis only in the U266-experiment when comparing the control group and groups with huPBMC (with and without Tregs). This cutaneous fibrosis was less extensive in mice the control group with U266-myeloma, but this effect was not seen in the experiment with the RPMI-8226 cell line.

A significant difference was seen between the murine control group and groups with huPBMC with and without additional Tregs for CD20+ B cells and CD138+ plasma cells in lung (U266 experiment) and liver (RPMI-8226 experiment). However, when comparing only the groups of huPBMC-injected mice with and without additional Tregs, only a significant difference in plasma cells in the liver was observed (RMPI-experiment), in which the number of plasma cells was significantly lower in mice receiving Treg-enriched huPBMCs.

The multiple myeloma cells ($CD138^+$) were morphologically different from the normal $CD138^+$ population (data not shown) and preferably localized in either femur or spine. A decrease in myeloma cells by huPBMC injection was observed histologically in spine for U266. There appeared to be lower numbers of plasma cells in spine too for RPMI-8226 in mice receiving huPBMC's, but was this was histologically not significant. No significant differences in number of Tregs were seen histologically in any of the evaluated organs between the groups with huPBMC with or without additional Tregs.

Table 2. Morphological analysis of tissues of mice injected with huPBMC with (+) / without (-) extra Tregs, in presence of myeloma.

	Experiment	N	Fibrosis	
		(A/B/C)	P value all (B vs. C)	significant
Spleen	U266	(3/5/4)	0.6081 (0.8963)	no
	RPMI-8226	(4/6/4)	0.9839 (1.000)	no
Lung	U266	(3/5/4)	no fibrosis detected	
	RPMI-8226	(4/6/4)	0.0666 (0.0922)	no
Liver	U266	(3/5/4)	0.3363 (0.4292)	no
	RPMI-8226	(4/6/4)	0.9365 (0.8783)	no
Skin	U266	(3/5/4)	0.0194 (0.2472)	**yes** (no)
	RPMI-8226	(4/6/4)	0.8544 (0.7348)	no
Femur	U266	(3/5/4)	not evaluated	
	RPMI-8226	(4/6/4)	not evaluated	
Spine	U266	(3/5/4)	not evaluated	
	RPMI-8226	(4/6/4)	not evaluated	

Groups are referred to as A. Control group injected with myeloma cell line only, B. mice injected with myeloma and huPBMCs or C. mice injected with myeloma, huPBMCs and additional Tregs.

B cells (CD20)		Plasma cells (CD138)		Tregs (FoxP3)	
P value all (B vs. C)	**significant**	**P value all (B vs. C)**	**significant**	**P value all (B vs. C)**	**significant**
0.1176 (0.8973)	no	0.2933 (0.8963)	no	0.0679 (1.000)	no
0.0205 (0.8170)	**yes** (no)	0.3704 (0.1694)	no	0.3548 (0.8231)	no
0.0330 (0.8676)	**yes** (no)	0.0439 (0.2918)	**yes** (no)	0.0874 (0.0942)	no
0.2135 (0.7600)	no	0.1276 (0.3961)	no	0.1684 (0.8783)	no
0.2484 (0.9110)	no	0.5862 (0.8952)	no	no Tregs detected	
0.0252 (0.0736)	**yes** (no)	0.0600 (0.0177)	no (**yes**)	0.2359 (0.3496)	no
0.5580 (0.8937)		no plasma cells detected		no Tregs detected	
no B cells detected		no plasma cells detected		no Tregs detected	
0.2457 (0.8231)	no	0.3256 (0.9029)	no	0.6456 (0.7609)	no
0.3953 (0.7835)	no	0.5387 (1.000)	no	0.6086 (0.8783)	no
0.6562 (0.3663)	no	0.0229 (0.7077)	**yes** (no)	0.2913 (0.3496)	no
0.1354 (0.9124)	no	0.0905 (0.2785)	no	0.1237 (0.5557)	no

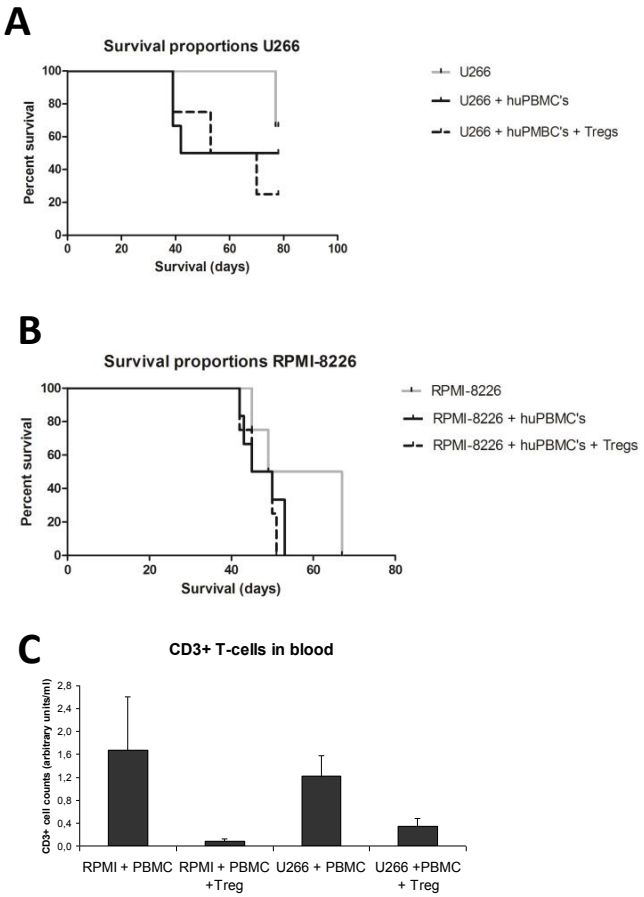

Figure 2. Effect of additional Tregs in RAG2$^{-/-}\gamma c^{-/-}$ mice, inoculated with either U266 multiple myeloma cell line or RPMI-8226 multiple myeloma cell line and injected with huPBMCs. A. Overall survival in mice with U266, B. overall survival in mice with RPMI-8226, C. CD3+ T cell counts in peripheral blood, showing the effects of additional Tregs in both luciferase labelled U266 and luciferase labelled RPMI-8226 inoculated mice. D. Bioluminescence imaging results showing luciferase activity in U266 inoculated mice. E. Bioluminescence imaging results showing luciferase activity in RPMI-8226 inoculated mice. Group sizes are shown in table 2.

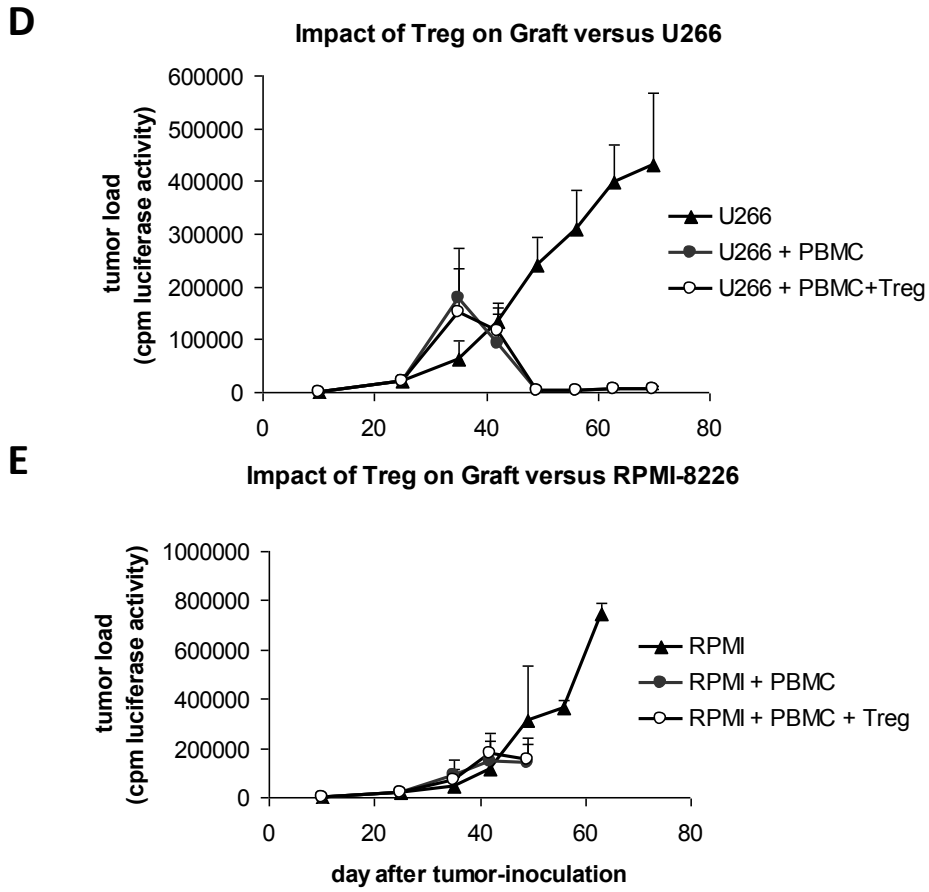

Figure 2. Continued.

DISCUSSION

In this study, we evaluated the importance of B cell activation with respect to its influence on Tregs (with regard to their presence and function) and their interaction with Tregs, in order to predict whether intervention on one of these cell-lines might be of interest in treatment of a GvHR.

B cell activation was analysed *in vitro*, in which presence of stroma cells was included to reflect a more natural environment. The *in vivo* study was considered the *in vivo* equivalent of our *in vitro* situation of stroma cells, Tresp and myeloma with and without additional Tregs.

In all *in vitro* experiments a significant stimulating effect of B cell activation on Tresp proliferation was noted. In half of the experiments a significant interaction between B cells and Tregs could be determined in which both Tresp and Tregs were affected by the B cells, making the significance of B cell stimulation of Tresp proliferation difficult to interpret. Tresp presence significantly enhanced Treg proliferation and an interaction between Tresp and B cells was shown only on occasion.

In the *in vivo* study the induction of a GvHR had a significant clinical effect on myeloma load with regards to luciferase activity. However, with the additional boost of Tregs, survival for the GvHR was not significantly improved. The significant reduction in lymphocyte counts in peripheral blood contrasted the rather unremarkable differences regarding the histological cell infiltrate analysis in several organs between huPBMC injected mice with and without additional Tregs. A histological significant reduction of myeloma in spine could be confirmed for the U266 myeloma cell line only. Similar to the RPMI-8226 myeloma cell line this histological reduction did however not correlate well with the results from bioluminescence imaging in which the effect was more profound, although one should realize that the BLI was performed total body and our histology was based on samples. A sampling error could have occurred.

Our *in vitro* results cannot exclude or confirm a suppressive effect of Tregs on B-cells as suggested by Lim *et al* [17], because an interaction was only observed in half of our experiments. A possible effect of match / mismatch between donor and host could possibly explain this, as in each of our *in vitro* experiments a different combination of donors was used, which was inevitable, but also important as it may indicate the importance of the type of mismatch between donor and recipient.

With regard to known literature on B cell activation, the *in vitro* findings contradict the findings of Shah *et al* [16], who suggested that expansion of Tregs is the result of inactivity of B cells. Our *in vitro* study showed significant proliferation of Tregs and Tresp in response to B cell activation. However, our *in vivo* study showed that in the presence of myeloma (regarding myeloma as the ultimate form of an activated B cell) the addition of extra Tregs in the Tresp proliferation could not be histologically confirmed in the organs and the addition of Tregs did not result in a significant decrease in GvHR induced sclerosis either. Perhaps the effect of B cell activity on infiltrating Treg numbers and sclerosis development *in vivo* is more functional and related to cytokines [25] rather than the morphological quantity of infiltrating cells within the organs. This could explain the minor histological changes observed in our morphological analysis, although two other hypotheses are also possible.

First, it could be that in our *in vivo* study the GvHR under influence of an activated B cell / plasmacytoid population developed too extensively to be stopped by the addition of Tregs in order to show a decrease in sclerosis development or second, the results might reflect an influence on Treg suppressive activity in the GvHR reaction by myeloma cells [18].

In our *in vivo* experiment, the addition of Tregs did not result in a histologically visualized increased infiltration of Tregs in any organ. Perhaps this is due to the presence of myeloma, or the number of Tregs is just too low to be properly assessed with light microscopy for small differences. However, we cannot exclude an influence of myeloma on Treg function or morphological cell quantity in our GvHR.

Although for U266 a graft-versus-tumour (GvT) effect appears to persist during the GvHR, for RMPI-8226 this effect could not be confirmed. A possible explanation could be, that this cell-line might grow more aggressively and the GvT might not be enough to compete with myeloma-growth in order to see a histological difference in tumour load, or this cell-line might have a different effect on the surrounding Tregs and other inflammatory cells compared to U266. Another possibility is that in the RPMI-8226 group mice might have died from GvHD before a GvT effect could be visualized.

Considering the pathogenesis of acute and chronic GvHD as proposed by MacDonald *et al*. [11] and our current findings of a virtually continuing GvHD damage within the organs, it would be more interesting to look at the complete cascade of GvHR rather than single cell-lines only in future research, preferably including the importance of e.g. TGF-β and other cytokines in order to explain the lack of correlation between clinical signs and histology.

To conclude, activation of B cells results in a more intense T cell proliferation and therefore may lead to a more intense GvH reaction. Tregs proliferate along with this reaction. Therefore, one might think that B cell depletion might be more effective when depletion is performed in a later stage or when more Tregs are added. However, our *in vivo* data show that once a reaction is established, turning the reaction around with additional Tregs is a very difficult task. Although the Treg function might be influenced by the presence of myeloma, additional Tregs did not result in a decreased GvHR in organs not affected by myeloma. An early depletion of B cells might be a better option and has been shown to be effective [11] but even then, the benefit might only be restricted to a clinical observation and might not necessarily reflect a decrease in histological organ damage. How we can actually stop histologically confirmed organ-damage from the GvHR by means of Treg or B cell intervention therefore remains an intriguing subject.

DECLARATIONS

Conflict of interest disclosure

The authors have no conflicts of interests to disclose.

Data availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

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CHAPTER 6

CLINICAL VERSUS HISTOLOGICAL GRADING IN THE ASSESSMENT OF CUTANEOUS GRAFT-VERSUS-HOST DISEASE

M.C.H. Hogenes[1], L.C.J. te Boome [2,3], D.C. van der Valk[3], M.R. van Dijk[4], R.A. de Weger [4], J. Kuball[3,5], van Diest P.J.[4]

[1]LABPON, Laboratory for Pathology East Netherlands
[2]MCH Haaglanden department Internal Medicine
[3]UMC Utrecht, department of Hematology
[4]UMC Utrecht, department of Pathology
[5]UMC Utrecht, Laboratory of Translational Immunology

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ABSTRACT

Skin biopsies are often used in daily practice for the diagnosis of acute (aGvHD) or chronic graft-versus-host disease (cGvHD). With the latest understanding in pathogenesis and new National Institute of Health (NIH) classifications for aGvHD and cGvHD, there is a need to evaluate the current prognostic value of histological grading cutaneous GvHD and its correlation to the clinical grade.

In a retrospective study with 120 skin biopsies (all taken for suspected GvHD) from 110 patients (all classified according to the NIH), biopsies were revised and graded, blinded for clinical information, for either acute of chronic features. Morphological grades were compared for concordance with the clinical grade and survival analyses were done for clinical and histological grading.

Correlation for histologic vs. clinical grading was (very) poor for aGvHD and cGvHD (weighted κ -0.038 and 0.0009, respectively). Patients with clinical aGvHD had worse prognosis compared to cGvHD. However, at time of biopsy neither clinical nor histological grading predicted the eventual survival for either aGvHD ($p=0.9739$ and $p=0.0744$, respectively) or cGvHD ($p=0.2149$ and $p=0.4465$, respectively).

Confirming the diagnosis of GvHD is still a valuable reason for taking a skin biopsy, but this study shows that histologic grading of GvHD in the skin biopsy has no additional value for clinicians in current practice.

INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is a potentially curative therapy with proven efficacy in the management of hematologic malignancies. However, it can be complicated by the syndromes of acute and chronic graft-versus-host disease. Graft-versus-host disease (GvHD) is a major cause of morbidity and mortality of HCT. Skin, liver, and intestine are regarded as the principal target organs of GvHD and can be affected to varying degrees or not at all.

Skin biopsies are often taken to differentiate cutaneous GvHD from other diseases with similar cutaneous symptoms. Although some reports question the value of skin biopsies since histological features may overlap with other skin diseases, they are by most still regarded essential in diagnosing aGvHD. For cGvHD their value is less clear.

To assess severity of cutaneous GvHD, several grading systems have been developed. These either have a clinical or histological point of view.

Clinically cutaneous cGvHD is either sub-classified as limited or extended [1] or as mild, moderate or severe cGvHD [2, 3]. New classification systems incorporate clinical symptoms and patient's functional status, but sometimes fail to include lichenoid features separately and mainly focus on sclerosis [2] or do not differentiate between both types within their grading system [3]. However, lichenoid and sclerotic cGvHD show different response to treatment, in which lichenoid cGvHD has a poor prognosis [4, 5].

In the histological evaluation of a skin biopsy, pathologists try to assist clinical decision makers by confirming the diagnosis GvHD and grade the morphological severity. The histologic criteria for diagnosing cutaneous GvHD include features for aGvHD as well as lichenoid and sclerotic cGvHD patterns [6]. For aGvHD, Horn's adapted Lerner grading system [7, 8] is often used. For cGvHD however, there are no strictly formulated histological criteria to evaluate severity of either lichenoid or sclerotic cGvHD.

Correlation studies between clinical and histological features have been performed previously for gastrointestinal biopsies [9-12] in which a correlation between clinical and histological grading has both been suggested [11] as well as denied [12]. In cutaneous GvHD a similar lack of correlation between clinical and histological grading has been reported [13], but that was long before the clinical manifestations and new classifications were taken into account.

In this study, we therefore analyzed the current prognostic value of skin biopsies in GvHD and whether histological grading of acute and chronic GvHD correlates with clinical manifestations.

MATERIAL AND METHOD

Patients selection and clinical data

According the JACIE and EBMT guidelines, transplantation data were collected from patients and donors after informed consent was signed in the allogeneic HCT unit of the Hematology Department of the University Medical Centre of Utrecht (UMCU). A search was performed in the local pathology database for patients diagnosed with GvHD based on a skin biopsy between April 2007 and September 2011. Cases with more than 1 skin biopsy during follow-up were treated as single individuals (n=110) for patient related statistics, and the skin biopsies were considered separate events in our analyses with histology (n=120). Patients were clinically classified according to the NIH definitions (6) as aGvHD (including classical aGvHD and persistent, recurrent or late onset aGvHD, after HCT or donor lymphocyte infusion) or cGvHD (including classical cGvHD –chronic progressive, quiescent or *de novo*- and overlap syndromes of cGvHD with aGvHD). Clinical grading of GvHD was done following the adapted grading system from Glucksberg [14] and Pzerpiorka [15] for aGvHD and the Seattle criteria proposed by Shulman *et al* [1] were used to distinguish limited and extensive cGvHD (table 1). In order to evaluate the percentage of skin involvement in cGvHD, percentages were chosen that are similar to the acute criteria. A summary of the baseline statistics of our patients can be found in table 3.

Revision of the clinical grading (for skin involvement as well as overall clinical grading) was performed based on the actual documented clinical symptoms and signs and compared to the original clinical grading for coherence.

Table 1. Clinical grading system for acute (A and B) and chronic (C) graft-versus-host disease.

A. Clinical grading acute GvHD*

grade per organ	Involvement skin: % body surface area	Involvement GI: volume diarrhea (ml/day)	Involvement liver: level billirubin (µmol/l)
0	no rash caused by GvHD	< 500	< 20
1	< 25%	500-1000; or nausea and vomiting with positive biopsy	20-40; with moderate increase in ASAT (150-170 IU)
2	25-50%	1000-1500; or more severe nausea and vomiting	40-75
3	> 50%	1500-2000; or more severe nausea and vomiting	75-200
4	> 50% with bullous formation or desquamation	> 2000; or severe abdominal pain with or without ileus	> 200

B. Total clinical grade acute GvHD*

Total grade aGvHD	Skin grade	GI grade	Liver grade	Clinical performance
0	0	0	0	Normal
1	1-2	0	0	Normal
2#	1-3	1	1	Mildy decreased
3#	2-3	2-3	2-4	Markedly decreased
4	2-4	2-4	2-4	Extremely decreased

* Grading adapted from Glucksberg *et al* [14] and Przepiorka *et al* [15].

C. Clinical grading chronic GvHDϕ

Grade	Criteria
Limited	Only involvement of skin and liver
Extensive	Involvement of skin, liver AND any other organ

ϕ Grading according to the Seattle criteria by Shulman *et al* [1].

Histological grading

All skin biopsies retrieved in this search were revised by two pathologists and graded blinded to clinical information for lichenoid (as a representation of acute/active) and sclerotic (as a representation of chronic) morphological features. The pathologists were unaware of the interval between biopsy and transplantation as well as the clinical symptoms and had to decide for acute and chronic features purely based on morphology of the skin biopsy. Revised grades were compared to the original grading at time of biopsy for coherence.

For the lichenoid/acute grading, Horn's criteria [7] were adapted (table 2). Due to current lack of official grading systems for sclerotic/chronic GvHD, we have developed a grading system focusing on limited or extensive sclerotic changes for the purpose of this study (table 2). The hypothesis used is that severity of sclerosis can be most objectively reflected as the extend of sclerosis into the deeper dermis, often resulting in loss of sub-epidermal fat and incorporation of adnexal structures of the skin.

Statistics

Coherence between clinical and histological grading was performed using kappa, weighed kappa and correlation between histology and clinical grading using Pearson's r test. Survival analyses were done by plotting Kaplan-Meier curves and the Log Rank test. All tests were done with Graphpad Prism version 5.0. P-values below 0.05 were regarded significant.

Table 2. Histological grading system for acute (A) and chronic (B) cutaneous graft-versus-host disease.

A. Histological grading of acute GvHD†	
Grade	**Morphological criteria**
0	Normal skin or epidermal changes due to other causes than GvHD
1	Vacuolar alteration of junction between epidermis and dermis
2	Grade 1 with dyskeratotic cells within the epidermis and/or hair follicle, infiltrate of lymphocytes within the dermis
3	Grade 2 with fusion of fasilar vacuoles to form clefts and microvesicles
4	Separation of epidermis from dermis
† According to Horn's adapted criteria from Lerner[7]	
B. Histological grading chronic GvHD (proposed criteria focusing on sclerotic featues only)**	
Grade	**Criteria**
Limited	Scerlosis within the superficial dermis, including coarsening of the fibres
Extensive	Sclerosis extending into the deep dermis or very dense sclerosis in any layer of the skin

** In histological grading the lichenoid variant of chronic GvHD was graded according to the components in acute GvHD.

RESULTS

Results of our analysis are summarized in table 3 (for patient statistics), table 4 (for biopsy statistics) and table 5 (comparison and correlation statistics). For each analysis in our patients and biopsy analysis, the number of available cases for evaluation from our data collection is provided.

Evaluation of patients

Table 3 shows that in 110 patients, mean survival was 2.96 years (SD 2.47), without significant effects on survival of gender, age range, disease, donor type, conditioning regiment and without a significant effect of infection after transplantation (which occurred in a high number of patients). Considering clinical GvHD, patient files were found to be fully documented for our topics of interest in 98.2% of our individuals, revealing that patients either were known clinically with aGvHD only, had both aGvHD as well as cGvHD or experienced a form of cGvHD without a preceding aGvHD. Survival between these groups was significantly different (p=0.0037). Separating these patients in more rigid groups (just acute and chronic GvHD, without considering the potential overlap of experiencing both at some time during follow up), 86.4% of patients had a documented form of aGvHD, of which 60.9% presented themselves having aGvHD when their skin biopsy was taken. Presenting clinically with an acute form of GvHD had a significant negative effect on survival, when compared to presenting clinically with cGvHD (p=-0.0036) and the clinical maximum skin-grade (the maximum percentage of skin involved in these patients) also showed significant differences in survival (p<0.0001).

81.4% of our patients were known with a documented chronic form of clinical GvHD, but the type of cGvHD presentation (based on the NIH classification) showed no differences in survival (p=0.44). The maximum overall clinical grade of cGvHD reached had no significant effect (p=0.8844) either, nor did the percentage of skin involved when looking at skin only (p = 0.7622). Even though clinical cGvHD in patients did not show significant differences in survival between the different forms of cGvHD, the clinical grade of cGvHD did correlate with survival (p=0.0002).

Evaluation of biopsies

When all skin-biopsies were considered a separate event (n=120), survival did not significantly differ with the percentage of skin involved at time of biopsy (p=1.668). However, survival was not significantly different when considering the overall acute clinical grade including all tracts involved either (p=0.4059). For patients presenting clinically as aGvHD at the time of biopsy, there was no effect of the skin grade (p=0.7702) or overall clinical acute grade on survival (p=0.6812). For patients with cGvHD at the time of skin biopsy, the overall clinical grade had no significant

effect (p=0.1062). Even though the Seattle criteria normally do not use severity of each tract involved, we also evaluated the effect of skin-percentage involved in these cases (as this is used when grading for aGvHD), but this did not influence survival either (p=0.5231).

Both patients with clinical aGVHD and clinically cGvHD at time of biopsy could show histological features of acute and chronic/sclerotic histological GvHD. There is no significant effect on survival for either patients with aGvHD (p=0.8111), nor for those with clinical cGvHD (p=0.7996) at presentation of their skin biopsy (figures 1 and 2 in supplementary data), nor for the histological grading at that time (figures 1 and 2 in supplementary data). In fact, there was no effect on survival at all when purely looking at acute histological features or chronic (sclerotic) features when comparing survival based on histological criteria only either (histological aGvHD n=117, p=0.9509 and histological chronic/sclerotic features (n = 115, p=0.829).

Comparing kappa scores for revised histological versus clinical grading, correlation was very poor for aGvHD (κ 0.014), even when adjusting for the degree of difference in grade (weighed κ -0.038) and poor for cGvHD (κ 0.015, weighted κ0.0009). There was no significant correlation between histological scoring and clinical scoring of both aGvHD as well as cGvHD (table 5 general statistics). Interobserver variability between both pathologists at revision of histology was very low for acute GvHD (κ 0.090 and weighted κ 0.945) as well as chronic GvHD (κ0.960 and weighted κ 0.968) and complete consensus for each histological grade was reached after consultation.

Table 3. Patients statitics (with survival comparison), each patient as single individual.

Patient statistics (with survival comparison)				
Feature	**grouping / specifics**	**frequency**	**%**	**p value**
Number of patients	N	110	100.0%	
Survival patients overall (years)	Mean	2.96 (SD 2.47)		
Gender	Male	72	65.5%	0.526
	Female	38	34.5%	
Age	Mean	45.9 (SD 17.5)		
	0-10	8	7.3%	0.328
	10-20	4	3.6%	
	20-30	8	7.3%	
	30-40	11	10.0%	
	40-50	19	17.3%	
	50-60	36	32.7%	
	60-70	24	21.8%	
Disease	Lymphoma	18	16.4%	0.3137
	Plasma cell disorders	23	20.9%	
	MDS/MPN	12	10.9%	
	Bone marrow failure	3	2.7%	
	Inherited disorders	6	5.5%	
	Metabolic disorders	1	0.9%	
	AML	25	22.7%	
	CML	3	2.7%	
	ALL	11	10.0%	
	CLL	8	7.3%	
Donor type	Sibling (SIB)	34	30.9%	0.1867
	Cord Blood (CB)	11	10.0%	
	Matched Unrelated Donor (MUD)	63	57.3%	
	Other	2	1.8%	
Conditioning type	Myelo ablative	17	15.5%	0.2449
	Non-myelo ablative	72	65.5%	
	Reduced intersity	21	19.1%	
Infection after treatment	Overall	95	86.4%	0.1582
	CMV	*32*	*29.1%*	*0.9485*
	HHV6	*10*	*9.1%*	*0.2073*
	EBV	*7*	*6.4%*	*0.6031*
	Aspergillus	*11*	*10.0%*	*0.2082*
	Candida	*31*	*28.2%*	*0.2318*

Table 3. Continued.

Patient statistics (with survival comparison)				
Feature	**grouping / specifics**	**frequency**	**%**	**p value**
Clinical GvHD	Overall (fully documented)*	108	98.2%	0.0037
	Acute GvHD only (1)	18	16.4%	
	Both acute and chronic GvHD (2)	77	70.0%	
	Chronic GvHD only (3)	13	11.8%	
Clinical acute GvHD (1+2)	Mean survival (years)	3.0 (SD 2.6)		
	Overall no. patients with documented acute GvHD*	95	86.4%	
	aGvHD presentation at date skin biopsy (with no. biopsies)	67 (68)	60.9%	0.0036
	Max. clinical documented aGvHD skin-grade reached	66		<0.0001
Clinical chronic GvHD (2+3)	Mean survival (years)	3.3 (SD 2.5)		
	Overall no. of patients with documented chronic GvHD*	90	81.8%	
	cGvHD presentation at date biopsy (with no. biopsies)	48 (52)	43.6%	0.44
	Progressive	*13 (15)*		
	Quiescent	*6 (7)*		
	Late onset aGvHD	*19 (20)*		
	De Novo	*4 (4)*		
	Late onset aGvHD after DLI	*6 (6)*		
	Max. clinical documented cGvHD grade reached (including all tracts)	48	43.6%	0.8844
	Max. clinical documented % skin involvement:	46	41.8%	0.7622
	0%	6		
	0-25%	15		
	25-50%	25		
	>50%	0		

Table 4. Patients statistics (with survival comparison), each biopsy being a single event.

Biopsy statistics (with survival comparison)				
Feature	**grouping / specifics**	**frequency**	**%**	**p value**
Number of biopsies	N	120	100.0%	
Clinical GvHD				
Overall (not aGvHD or cGvHD specified)	Graded based on % skin involvement	117	97.5%	1.668
	no GvHD (0%)	*1*		
	Grade 1 (<25%)	*24*		
	Grade 2 (25-50%)	*57*		
	Grade 3 (>50%)	*35*		
	Grade based on involved tracts	*118*		0.4059
	Limited (Only skin and liver)	*67*		
	Extensive (involvement of skin, liver AND any other organ)	51		
Clinical acute GvHD at date biopsy	Overall grade (all tracts included)	66	55.0%	0.6812
	no aGvHD	*1*		
	grade 1	*10*		
	grade 2	*33*		
	grade 3	*23*		
	grade 4	*0*		
	Grade skin	*66*	55.0%	0.7702
	no aGvHD	*0*		
	grade 1	*7*		
	grade 2	*59*		
	grade 3	*24*		
	grade 4	*0*		
Clinical chronic GvHD at date biopsy	Overall grade (all tracts included)	48	40.0%	0.1062
	Limited	*15*		
	Extensive	*33*		
	"Grade" skin (% skin involvement)	48	40.0%	
	No skin involvement	*0*		0.5231
	<50% skin involved (limited)	*35*		
	>50% skin involved (extensive)	*13*		
Histological GvHD				
Histological acute GvHD in biopsy	Histological acute grade (Horn)	*117*	97.5%	0.9509
	no aGvHD	*8*		
	grade 1	*47*		
	grade 2	*54*		
	grade 3	*8*		

Table 4. Continued.

Biopsy statistics (with survival comparison)				
Feature	**grouping / specifics**	**frequency**	**%**	**p value**
Histological chronic GvHD in biopsy	Histological chronic/sclerosis grade	*115*	95.8%	0.829
	No sclerosis	*10*		
	Limited	*30*		
	Extensive	75		
Biopsies of patients presenting with clinical aGvHD	Histological acute grade (Horn)	66	55.0%	0.8111
	no aGvHD	*3*		
	grade 1	*25*		
	grade 2	*34*		
	grade 3	*4*		
	Histological chronic/sclerosis grade	*64*	53.3%	0.5068
	No sclerosis	*7*		
	Limited	*16*		
	Extensive	41		
Biopsies of patients presenting with clinical cGvHD	Histological acute grade (Horn)	46	38.3%	0.8306
	no aGvHD	*5*		
	grade 1	*20*		
	grade 2	*17*		
	grade 3	*4*		
	Histological chronic/sclerosis grade	*46*	38.3%	0.7996
	No sclerosis	*3*		
	Limited	*14*		
	Extensive	29		

Table 5. General statistics with kappa and correlation evaluation between clinical and histological grading of acute and chronic (cutaneous) GvHD.

Feature				
Number of biopsies			N	120
Kappa score evaluation				
	comparing features		kappa	weighed kappa
Histology vs clinical	Revised histological aGvHD	Revised clinical aGvHD	0.014 (poor)	-0.038 (very poor)
	Revised histological cGvHD	Revised clinical cGvHD	0.015 (poor)	0.0009 (poor)
Histology	Revised histological aGvHD	Original histological aGvHD grade	0.353 (fair)	0.391 (fair)
	Revised histological cGvHD	Original histological cGvHD grade	*not possible (no original grades)*	
Clinical	Revised clinical aGvHD	Original clinical aGvHD grade	0.526 (moderate)	0.489 (moderate)
	Revised clinical cGvHD (all tracts)	Original clinical cGvHD grade (all tracts)	0.909 (very good)	0.829 (very good)
Correlation evaluation				
	comparing features			p value
Biopsies from all patients	Revised histological score aGvHD	Revised clinical aGvHD		0.6364
	Revised histological score cGvHD	Revised clinical cGvHD overall		0.7843
	Revised histological score cGvHD	Revised max. clinical cGvHD score skin *Limited 0-50% skin involvement* *Extensive >50% skin involvement*		0.2382
Biopsies from aGvHD patients	Revised histological aGvHD	Revised clinical aGvHD		0.3409
	Revised histological aGvHD	Max. documented clinical aGvHD		0.3498
	Revised histological aGvHD	Survival		0.0744
	Revised clinical GvHD	Survival		0.9739

Table 5. Continued.

Correlation evaluation			
	comparing features		p value
Biopsies from cGvHD patients	Revised histological cGvHD	Revised clinical cGvHD overall	0.5105
	Revised histological cGvHD	Revised clinical cGvHD skin	0.157
		Limited 0-50% skin involvment	
		Extensive >50% skin involvement	
	Revised histological cGvHD	Revised max. clinical cGvHD score skin	0.157
		Limited 0-50% skin involvment	
		Extensive >50% skin involvement	
	Revised histological cGvHD	Survival	0.4465
	Revised clinical cGvHD overall	Survival	0.0002
	Revised max. clinical cGvHD score skin	Survival	0.1249
	Limited 0-50% skin involvment		
	Extensive >50% skin involvement		

DISCUSSION

The aim of this study was to evaluate the value of histological grading of cutaneous GvHD and to compare its correlation to the clinical grading of cutaneous GvHD. In summary, our results confirm the lack of correlation between histological and clinical grading of both acute as well as chronic GvHD. For survival, all that seems to matter is the presence of clinical aGvHD and the percentage of skin involved when having aGvHD in skin, while histologic grading of acute and chronic GvHD at the time of biopsy had no prognostic value. The results however should be regarded with caution, for several reasons.

First: as GvHD is a multi-organ disease, predicting survival on cutaneous GvHD alone does not reflect the importance of GvHD symptoms in other involved tracts for a patient's prognosis. This could explain the lack of prognostic value for histological grading of a skin biopsy only.

Second: as our cases were selected based on having a skin biopsy with GvHD, we do have certain selection bias in our study. Most of these skin biopsies will only have been taken when diagnosing cutaneous GvHD was clinically difficult. The small number of histologically grade III aGvHD in our study can be considered a reflection of this issue. Biopsies could have been taken early in the development of the disease and grade might progress after the biopsy was taken. We cannot be sure that histological grading of skin biopsies makes no sense whatsoever if a skin biopsy would be taken *at all* times. However, in our current daily practice in which biopsies are usually just performed to confirm clinical suspicion early and need to be justified in view of costs and morbidity, grading GvHD in a biopsy to predict survival has no value at this moment.

Third, treatment (after histologically establishing GvHD) has a considerable beneficial effect on survival of the individual GvHD patient which may obscure the natural adverse course of histological high grade GvHD.

Last, grading chronicity of GvHD using sclerosis is rather complex due the lack of existing grading systems for cGvHD, but also confounding other causes for sclerosis.

To address the grading issue for cGvHD, we proposed a grading system using the extension of sclerosis to the deeper dermis as a reflection of severity. This might not fully reflect the natural progression of a cutaneous cGvHD, but in our opinion is the most objective way to evaluate sclerosis. Unfortunately, deeper dermal involvement might be missed when a biopsy was too superficial. That might have influenced the results of our analysis.

We noticed that in many cases of clinical aGvHD, the biopsies often already morphologically showed a certain degree of sclerosis. It is possible that in daily practice this fact is overlooked and therefore not recognized or mentioned in reports. A plausible hypothesis, apart from co-existence of delayed aGvHD in patients with existing sclerodermatous cGvHD or a combination of clinically both lichenoid and sclerotic cGvHD, is that sclerosis histologically might develop in patients with aGvHD even before clinical symptoms of cGvHD occur. However, confounding causes for sclerosis cannot be excluded. A possible effect of immunosuppressive treatment in the formation of dermal fibrosis have been previously addressed by Shulman *et al*. [6]. Their report also addresses the difficult overlapping features between active cGvHD and aGvHD that might influence the interpretation of a skin biopsy as well as the risk of false-negative results when a biopsy is taken too soon after developing symptoms. It also refers to false-positive results due to recurrent infections, drug reactions or other inflammatory reactions, although not all infections will influence survival [16]. In other words, the presence of sclerosis in a skin biopsy is subject to many causes and therefore, using sclerosis to grade cGvHD remains difficult.

The design of our study underscores the importance of clinical information at time of biopsy. In our study the skin biopsies where histologically graded blinded to clinical information, therefore the interpretation was at risk for false-positive or false-negative results. Skin biopsies diagnosed as aGvHD in our study might in fact have been active cGvHD cases. In addition, the presence of collagen in the dermis is subject to the biopsy location, so lack of information on biopsy site might influence the grading of the sclerodermatous components[6]. We feel that by referring these cases as having aGvHD features in our study, the activity and possible implications to the clinical features compared to a more sclerotic reaction are still properly addressed. Nevertheless, in our study neither acute nor chronic histological features appeared to influence patient survival.

Our study was retrospective and we noticed that in the original grading of the disease both clinically as well as histologically, criteria were not always used correctly. This reflects the rather cumbersome and difficult staging and grading criteria, which are frequently not accurately followed outside clinical trials. Our revised data correlate very well for clinical cGvHD cases, moderately for clinical aGvHD cases, but only fairly for aGvHD features. In order to tackle this issue, our evaluation was based on the documented clinical features to revise clinical grade and a blinded revision of histology. Performing a prospective study in which both clinicians and pathologists are restricted to official grading standards (and perhaps applying double reading) is nevertheless highly recommended to confirm our current results.

In conclusion we feel that at present skin biopsies in daily practice serve no other purpose than to confirm or deny the clinical diagnosis GvHD when in doubt. As the histologically acute features and sclerotic features do not restrict themselves to an acute or chronic group of patients and the presence of cutaneous GvHD alone does not reflect the importance of other involved organs systems either for the survival of a patient, we recommend to stop classifying the histological findings in a skin biopsy into acute or chronic in our pathology reports and just lump the histological diagnosis into one group of 'histological findings consistent with GvHD' with either lichenoid or sclerodermatous features, leaving the sub classification into acute or chronic presentation to our clinicians. Histological grading of a skin biopsy on its own does not predict a patient's survival and should be used either with lots of caution or preferably not at all to avoid incorrect assumptions on prognosis.

DECLARATIONS

Compliance with Ethical Standards

All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards

Consent for publication

Not applicable.

Data availability

The datasets generated and analyzed during this current study are available from the corresponding author on reasonable request.

Competing interests

The authors have no conflicts of interest to disclose.

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Not applicable.

SUPPLEMENTARY DATA

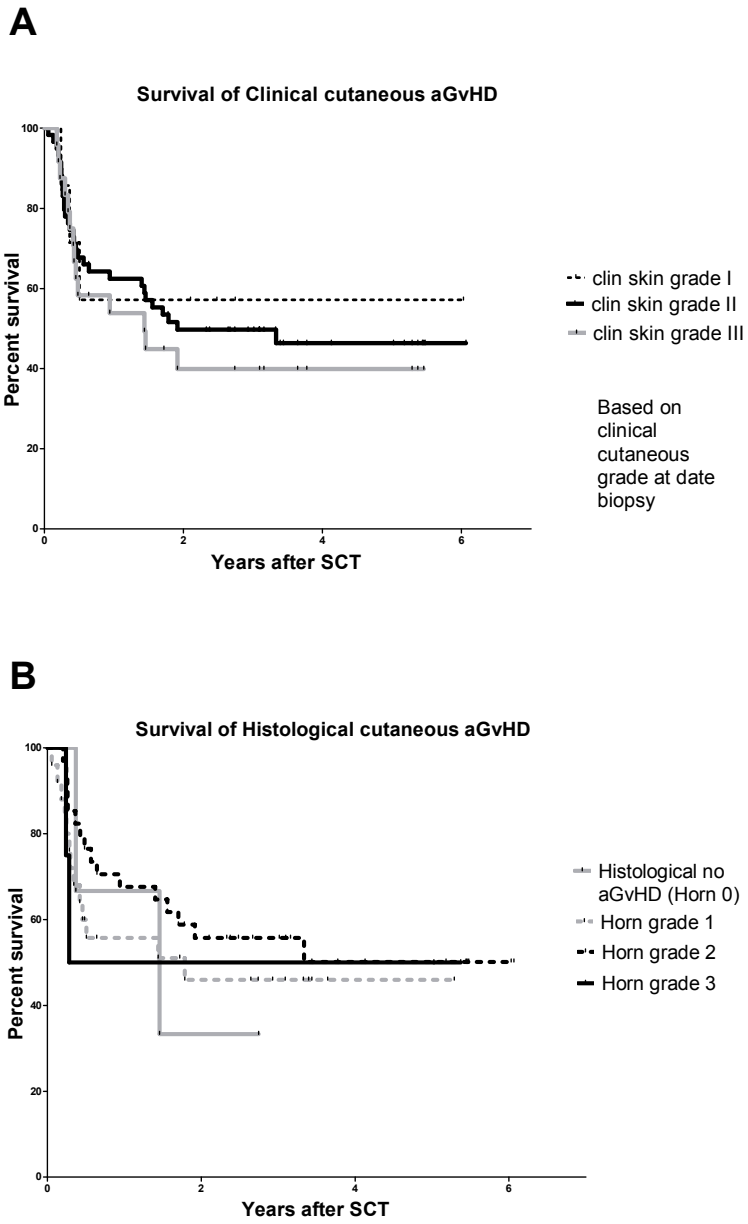

Figure 1. Survival proportions of acute cutaneous GvHD in patients presenting as acute GvHD at time biopsy (n=66). A. Survival comparison of acute clinical cutaneous GvHD grading (p=0.6812). B. Survival comparison of histologically acute GvHD grade in skin biopsies (p=0.8111).

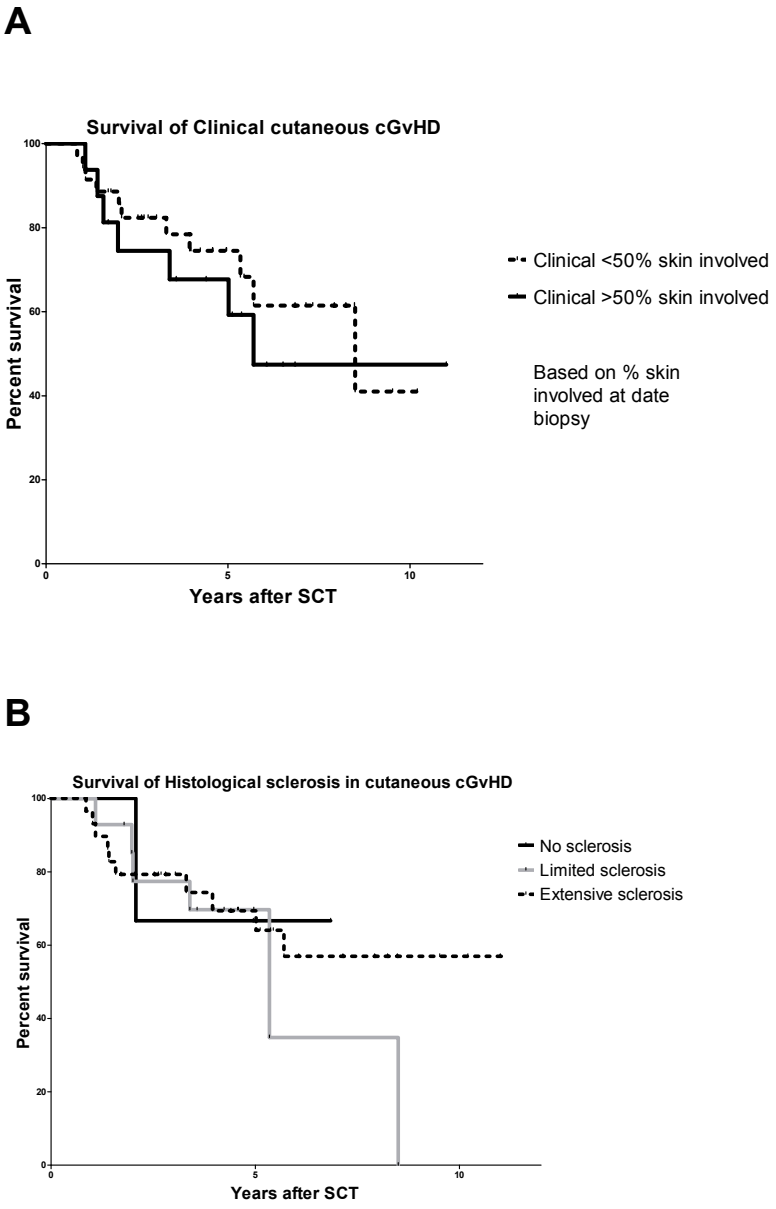

Figure 2. Survival proportions of chronic cutaneous GvHD in patients presenting as chronic GvHD at time of biopsy (n=48). A. Survival comparison between clinical percentage of skin involved (p=0.5231). B. Survival comparison of histologically chronic GvHD grade in skin biopsies (p=0.7996).

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CHAPTER 7

SUMMARIZING DISCUSSION

SUMMARIZING DISCUSSION

Morbidity and mortality are major concerns in graft-versus-host disease (GvHD), a response often seen after hematologic cell transplantation (HCT). Despite efforts in e.g. HLA-matching, graft-depletion of T cells and use of several immune-suppressive agents (including promising results for B cell depletion), GvHD remains a challenging disease to treat.

In this thesis we tried to contribute a small piece to the puzzle of GvHD pathogenesis by analyzing the interaction between B cells and regulatory T cells (Tregs), from a primarily histological perspective.

Highlights from this thesis

As indicated in **chapter 2**, humanized mice may serve as promising models in studies to graft-versus-host disease. Designing the right model for the topic of interest is however subject to many choices; one has to choose a suitable mouse strain from the extended range of (different immune deficient) mice available, choose the source of material to transfer, the route of transferal and discuss the possible use of additional pre- and posttreatment options before getting started. Based on our evaluation of the known pearls and pitfalls from different mouse strains in literature, we concluded that for our study to graft-versus-host disease, a xenogeneic $RAG2^{-/-}\gamma c^{-/-}$ mouse model by intravenous transfer of human peripheral blood cells (huPBMC's) after total body irradiation was a sensible choice.

The basic kinetics and pathology of our chosen xenogeneic mouse-model was outlined in **chapter 3**, addressing not only the obtained clinical symptoms of the graft-versus-host response like weight loss, decreased mobility, ruffled fur and skin erythema but also the cytokine landscape within several organs, indicating an interesting interplay amongst human B cells, human T cells and mouse macrophages at the level of human TGF-β and IFN-γ with murine CTGF with respect to the formation of fibrosis. Despite the difference in species used, the obtained histological assessment showed many similarities with the sclerotic variant of human chronic GvHD, though in our xenogeneic mice the preferred targets appear to be spleen, lung, liver and skin and does not include gut, in contrast to human GvHD.

After the clear visualization of the basic pathologic features of the graft-versus-host response in our xenogeneic model, **chapter 4** further addressed additional modifications to this model with either macrophage depletion or early B cell depletion.

Macrophage depletion is often used in humanized mouse models for GvHD to accelerate the induction of a response in mice. Our analysis confirms this acceleration, but also indicates that this potentially influences the composition of the cell-infiltrate within the targeted organs, often showing a significant increase in the number of B cells and plasma cells in spleen, lungs and liver and occasionally skin. Although the formation of fibrosis in our histologic evaluation was not significantly altered, some organs showed significant differences in their cytokine profile. Despite the increase in B cell quantities after macrophage depletion and plasma cell quantities, changes with regard to Tregs were limited to increased FoxP3 expression in spleen and bone marrow (though lacking histological confirmation of increased Treg infiltration within these organs).

The effect of macrophage-depletion to the severance of clinical symptoms, might suggest resemblances between the obtained reaction and acute GvHD in humans. It is known that host macrophages are related to the occurrence of GvHD, though donor macrophages are more likely to be related to the severity of the GvHD. It is possible that the fragments of tissue-damage from our conditioning regiment prior to huPBMC transfer might be preserved longer due to the depletion of macrophages, resulting in a possibly boosted cytokine related response. Perhaps the encountered increased numbers of B cells in our study, reflect a prolonged survival for their ability to function as antigen presenting cells as seen in the pathogenesis of acute human GvHD according to today's concepts.

Early B cell depletion without additional macrophage depletion in our xenogeneic model confirmed the signs of clinical improvement of GvHD symptoms as described previously by others. However, our histological evaluation contradicts a decrease in visceral organ damage. In fact, it is more likely that the encountered clinical improvement is related to TGF-β and liver fibrosis only. The unexpected lack of histological improvement in our early B cell depletion might reflect the importance of cytokines involved in the early GvH reaction.

In **chapter 5** we continued our exploration with the evaluation of B cell activation in a GvHD setting. Activating B cells *in vitro* showed a significant increase in T cell proliferation, but it also significantly boosted Treg proliferation. One could estimate that in the presence of activated B cells, Tregs are not effective enough to impair the extensive proliferation of responder T cells. To our surprise, introducing additional Tregs in our *in vivo* model with a GvH response in the presence of myeloma did not result in a diminished histological damage, despite clinical improvement. Again, this might reflect an important role of cytokines in the explanation of this contradiction between histology and clinical outcome as it is known that

the presence of myeloma may directly influence levels of some inflammatory cytokines. Some of these cytokines might still favor the formation of fibrosis, when a decrease in others may lead to a decrease in clinical symptoms.

In **chapter 6** we switched to human beings, to discuss the correlation (or the lack of it) between clinical and histological grading for cutaneous GvHD. We showed that in human cutaneous GvHD histological grading by itself is not sufficient to predict prognosis in current practice. Many confounders can influence in the interpretation of histologic findings. This knowledge underscores the difficulties of histology in the assessment for clinical prognosis. Development of a new grading system to predict prognosis in human GvHD is highly recommended.

Conclusions and considerations

With regard to the aim of this thesis three major conclusions can be drawn, which will be further addressed below:

1. B cells and Tregs in GvHD have a complex relation and their relation is subject to the activation of B cells.
2. Cytokines may explain the lack of correlation between histology and clinical symptoms of the GvHD reaction, as even the increase of a single cytokine in one organ (like TGF-β in liver) may result in an overall malaise dominating the clinical symptoms.
3. Rituximab serves best in the treatment for GvHD when used as an early B cell depletion therapy.

B cells and Tregs in GvHD

As indicated above we can confirm that B cells can play different roles in GvHD depending on their activation status. Their relation to Tregs also varies with their activation status. Early B cell depletion (likely depleting primarily resting B cells), improves clinical GvHD symptoms, probably by deleting the important APC-function of B cells from the equation. This does not result in changes in the histological appearance of disease though and this favors the importance of a cytokine-driven response in acute GvHD.

Low FoxP3 levels in lung and spleen after early B cell depletion might indicate a decrease in Treg-numbers, although histology could not confirm this. Therefore, a significant interaction between resting B cells and Tregs cannot be excluded, unless it reflects interactions on a functional instead of quantitative level at this phase.

Activation of B cells however, is associated with a proliferation of Tregs. The inhibiting function of Tregs on the Tresp proliferation induced by the activation of B cells is however not sufficient enough to keep the T cell proliferation and tissue damage under control. Once the reaction is started and reactive B cells may further develop in functional plasma cells, anti-CD20 targeting with Rituximab in our opinion no longer make sense. In the setting of plasma cells (in our study represented by myeloma) however, adding additional Tregs to the equation does not result in histological improvement, but improved clinical survival may be encountered nevertheless.

The missing link between histology and clinical outcome

Our study of human biopsies from cutaneous GvHD showed that grading is very hard to handle in clinical practise and histological grade often does not correlate to the clinical symptoms. Based on our results, we must conclude that histology by itself may not be the Holy Grail to predict a patients prognosis. Histology is subject to many influences in human, for example drug reactions, recurrent infections or even the timing of the biopsy. The complexity of histological GvHD grading for clinical purposes is therefore much more difficult to handle and use in human than it is in mice. Despite these difficulties in human GvHD we believe that in our mice the issue of the possible confounders as indicated in humans is not applicable. However, we discovered that even in our mice the histological findings did not always correlate with the clinical outcome.

Based on the analyses in our mice, we believe that the importance of cytokines in inducing clinical effects might be the missing link in the explanation of these differences between histological versus clinical severity of disease. We should therefore, consider to include cytokine development and -actions as a separate factor contributing to the severity of the clinical GvH response when developing a new grading system for human GvHD. A collaboration between pathologists and clinicians towards developing a single, overall grading system for human GvHD instead of separate systems for pathology and clinical use would be ideal.

Rituximab treatment in GvHD

Finally, we would like to address the current use for Rituximab in GvHD using the considerations that come forward from our analyses on B cells and Tregs as well as our ancillary findings with regards to histology versus clinical outcome.

Like with cutaneous biopsies, timing is essential with regard to B cell depleting therapy using Rituximab. Timing this therapy is essential in order to diminish GvHD symptoms. We must realize

though, that improvement is a subjective concept in GvHD. Despite clinical improvement to Rituximab, the use of this pharmakon may unfortunately not be able to completely prevent the occurrence of histologic damage.

With the lessons learned from this thesis, doctors will be forced to define their interpretation of 'harm' in the Hippocratic Oath and should decide on their primary goal of this therapy: do we treat the patient's symptoms or do we wish to actually stop vital organ damage from happening? How one decides to define "harm" in the setting of GvHD after HCT, will eventually be decisive in the doctors ability to keep the oath they once made ….

CHAPTER 8

NEDERLANDSE SAMENVATTING

NEDERLANDSE SAMENVATTING

Morbiditeit en mortaliteit zijn belangrijke problemen in graft-versus-host ziekte (GvHD), een reactie die vaak wordt gezien na een hematologische cel transplantatie (HCT), waarbij de graft (de donor) reageert tegen de host (de ontvanger). Ondanks pogingen door middel van bijvoorbeeld HLA-matching, het wegvangen van T-cellen uit de donorpopulatie en het gebruik van verscheidene immuun-remmers (inclusief de veelbelovende resultaten van B-cel depletie als gevolg van het middel Rituximab), blijft het een uitdaging om GvHD te behandelen.

In deze thesis hebben we geprobeerd een klein stukje van de pathogenese-puzzel van GvHD op te lossen, door de interactie tussen B-cellen en Tregs te analyseren vanuit een primair histologisch oogpunt.

Highlights uit deze thesis

Zoals aangegeven in **hoofdstuk 2**, zijn gehumaniseerde muizen veelbelovend als model om GvHD te bestuderen. Echter, voor het ontwikkelen van een goed model om een specifiek aandachtspunt binnen deze ziekte goed te kunnen bestuderen moet men veel keuzes maken; men moet een bruikbare muizen stam kiezen uit de diverse verschillende (immuun deficiënte) muizen stammen die er bestaan, bepalen welk materiaal men wil transplanteren, beslissen via welke route de transplantatie moet plaatsvinden en tevens bepalen of er vooraf of na de transplantatie nog additionele behandelingen plaats moeten vinden. Op basis van onze evaluatie van de bekende methodes en voor- en nadelen van verschillende muizen stammen in de literatuur, hebben wij geconcludeerd dat voor onze studie naar GvHD een xenogeneisch muismodel een goede keuze is en we hebben daarbij gekozen voor RAG2$^{-/-}$γc$^{-/-}$ muizen waarin intraveneus humane perifere bloedcellen (huPBMC's) worden ingebracht na bestraling van de gehele muis.

De basis van de ontwikkelende reactie in ons gekozen muis model wordt beschreven in **hoofdstuk 3**, waarbij niet alleen gekeken wordt naar de klinische symptomen van de graft-versus-host reactie die de muizen ontwikkelen (zoals gewichtsverlies, verminderde beweeglijkheid, doffe vacht en roodheid van de huid), maar waarbij ook gekeken wordt naar de aanwezigheid van diverse cytokines in de verschillende organen. Hieruit blijkt dat er een belangrijke interactie/samenwerking blijkt plaats te vinden tussen humane B-cellen, humane T-cellen en muis-macrofagen op het niveau van humane TGF-β en IFN-γ met muis CTGF als het gaat om het ontwikkelen van fibrose. Ondanks dat in het model verschillende species worden gebruikt als donor en ontvanger, laat de histologische analyse zien dat er veel overeenkomsten zijn met de sclerotische variant van humane chronische GvHD, hoewel in onze xenogeneische muizen met

name de milt, long, lever en huid worden aangedaan en de darm (in tegenstelling tot humane GvHD) in ons model ongemoeid blijft.

Na deze beschrijving van de basale pathologische kenmerken van de GvH reactie in ons muismodel, wordt in **hoofdstuk 4** gekeken naar de effecten van additionele aanpassingen in het model in de vorm van macrofaag-depletie en (vroege) B-cel depletie.

Macrofaag-depletie door het injecteren van clodronaat-bevattende liposomen is een veelgebruikte toepassing in diverse gehumaniseerde muismodellen voor GvHD, waarbij de GvH reactie in de muizen sneller / ernstiger ontwikkelt. Onze analyse bevestigt deze versnelde ontwikkeling, maar laat bovendien zien dat macrofaag-depletie mogelijk de samenstelling van de infiltrerende cel populatie in de diverse aangedane organen kan veranderen. Hierbij wordt een significante toename gezien in het aantal B-cellen en plasmacellen in de milt, longen en lever en soms de huid. Hoewel de macrofaag-depletie niet leidt tot een duidelijk verschil in fibrose ontwikkeling, is er wel een significant verschil in cytokines in de organen.

Het effect van macrofaag depletie op de ernst van de symptomen, doet een vergelijking overwegen tussen de reactie in onze muizen en acute GvHD in mensen. Het is mogelijk dat brokstukken van de weefselbeschadiging als gevolg van de bestraling voorafgaand aan de injectie van de huPBMC's langer in het lichaam aanwezig zijn, waardoor mogelijk de cytokine respons extra wordt gestimuleerd. Misschien dat het toegenomen aantal B-cellen in deze studie een uiting zijn van verlengde overleving van deze B-cellen om daarbij als antigeen presenterende cellen te kunnen fungeren, zoals dat ook volgens de huidige ideeën omtrent de pathogenese van acute humane GvHD wordt verwacht.

Ondanks de toename in het aantal B-cellen en plasmacellen na macrofaagdepletie, worden er weinig veranderingen gezien in het aantal aangetroffen Tregs. Er is enkel op mRNA een verhoogde FoxP3 expressie in milt en beenmerg, maar histologische bevestiging van een toegenomen Treg-infiltratie in deze organen ontbreekt.

Vroege B-cel depletie zonder additionele macrofaagdepletie laat in ons model dezelfde klinische verbetering zien qua symptomen als in de literatuur beschreven wordt. Desalniettemin laat onze evaluatie zien dat er geen afname is in histologische orgaanschade. In de praktijk lijkt het veel waarschijnlijker dat de klinische verbetering eerder gerelateerd is aan een effect van enkel TGF-β en lever fibrose. Het onverwacht ontbreken van duidelijke histologische verbetering in onze vroege B-cel depletie in overige organen onderstreept de rol van cytokines in de vroege GvHD reactie.

Hoofdstuk 5 gaat verder met de evaluatie van B-cel activatie in een GvHD setting. Activatie van B-cellen *in vitro* resulteert in een toename van T-cel proliferatie, maar geeft tegelijkertijd ook een boost aan de Treg proliferatie. Men zou kunnen concluderen dat in de aanwezigheid van geactiveerde B-cellen de Tregs niet effectief genoeg zijn om de explosieve proliferatie van reagerende T-cellen (Tresponders) tegen te gaan. Tot onze verbazing toont het *in vivo* model wel een klinische verbetering als er extra Tregs worden ingespoten bij een GvH reactie in bijzijn van myeloom, maar is deze verbetering wederom niet zichtbaar op histologisch niveau. Opnieuw suggereert dit een mogelijk belangrijke rol van cytokines in de verklaring van de contradictie tussen klinisch beeld en histologie, aangezien bekend is dat multiple myeloom zelf een direct effect kan hebben op de aanwezige inflammatoire cytokines. Daardoor is het mogelijk dat sommige cytokines nog steeds fibrose induceren, terwijl anderen meer een effect kunnen hebben op de klinisch zichtbare symptomen zoals algehele malaise.

In **hoofdstuk 6** wordt een uitstap gemaakt van muizen naar mensen, met betrekking tot de correlatie (of juist het ontbreken hiervan) tussen klinische en histologische gradering van humane cutane GvHD. In dit hoofdstuk wordt aangetoond, dat voor humane cutane GvHD in de huidige praktijk histologische gadering alleen niet in staat is om de prognose van een patiënt te voorspellen. Dit komt door verscheidene confounders die de interpretatie van de histologische bevindingen beïnvloeden. Deze bevinding laat zien waarom gebruik van histologie voor het inschatten van de klinische prognose zo lastig is. Voor humane GvHD is het ten zeerste aan te raden dat er een nieuw graderingssysteem wordt ontwikkeld om de prognose van patiënten goed te kunnen voorspellen.

Conclusie en overwegingen

Met betrekking tot het doel van deze thesis kunnen er drie belangrijke conclusies worden getrokken, welke elk daaronder nader worden toegelicht:

1. B-cellen en Tregs hebben in GvHD een complexe relatie en deze relatie is onderhevig aan de activatie status van de B-cellen.
2. Cytokines kunnen mogelijk het ontbreken van de correlatie tussen histologie en de klinische symptomen in de GvH reactie verklaren. Zo kan een de stijging van een enkele cytokine in 1 specifiek orgaan (zoals TGF-β in de lever) al leiden tot een algehele malaise die de klinische symptomen kan domineren.
3. Rituximab werkt waarschijnlijk het best in de behandeling van GvHD als deze wordt ingezet als vroege B-cel depletie therapie.

B-cellen en Tregs in GvHD

Zoals hierboven aangegeven kunnen we bevestigen dat B-cellen diverse rollen kunnen spelen in GvHD, waarbij de rol die gespeeld wordt afhangt van de activatie status van de B-cel. De relatie van de B-cellen tot Tregs varieert ook met hun activatie status. Vroege B-cel depletie (waarbij waarschijnlijk met name rustende B-cellen worden weggevangen) geeft een verbetering in klinische GvHD symptomen, waarschijnlijk omdat de belangrijke antigeen presenterende functie van de B-cellen in de reactie wordt uitgeschakeld. Dit geeft echter geen veranderingen in ernst van de histologische kenmerken van de ziekte en suggereert daarmee een belangrijke rol van een cytokine-gedreven respons in acute GvHD.

Lage waarden voor FoxP3 in long en milt na vroege B-cel depletie suggereren een afname in het aantal Tregs in deze organen, maar histologisch kon dit niet worden bevestigd. Daarmee kan een significante interactie tussen rustende B-cellen en Tregs niet worden uitgesloten, tenzij deze waarneming getuigt van een meer functionele dan een kwantitatieve relatie tussen deze cellen.

De activatie van B-cellen is juist geassocieerd met een proliferatie van Tregs. De remmende functie van Tregs op de Tresponder cellen die geïnduceerd worden door de activatie van de B-cellen is echter niet voldoende om te voorkomen dat deze Tresponder cellen gaan delen en orgaanschade optreed. Als de reactie eenmaal gaande is en reactieve B-cellen verder ontwikkelen naar functionele plasmacellen, heeft anti-CD20 therapie ons inziens niet langer zin. In de setting van plasmacellen (in onze studie in de vorm van myeloom cellen) leidt het toevoegen van extra Tregs echter evenmin tot een histologische verbetering, ondanks dat er wel een betere klinische overleving wordt waargenomen.

De ontbrekende schakel tussen histologie en klinische uitkomst

Onze studie met humane biopten van cutane GvHD toont dat graderen in de klinische praktijk te moeilijk te hanteren is en dat de histologische gradering vaak niet overeenkomt met de ernst van de klinische symptomen. Met onze huidige resultaten moeten we concluderen dat histologie alleen niet het gouden ei is waarmee de prognose van een patiënt kan worden voorspeld. Histologie is immers onderhevig aan diverse invloeden van bijvoorbeeld medicatie-gebruik, terugkerende infecties of zelfs maar de timing van het biopt. Dit maakt histologische gradering van cutane GvHD in mensen dan ook vele malen lastiger te hanteren dan in een muismodel. We veronderstellen dat in ons muismodel deze interrumperende invloeden niet van toepassing zijn. Desalniettemin zien we ook in onze muizen dat de histologie niet altijd correleert met het klinische plaatje.

Op basis van onze muis bevindingen, denken wij dat cytokines een belangrijke rol spelen en daarmee de missende schakel vormen tussen de histologie versus de klinische ernst van de ziekte. We moeten daarom overwegen om de cytokine ontwikkeling en -acties mee te nemen als onafhankelijke factor bijdragend aan de ernst van de klinische GvHD respons als er een nieuw graderingssysteem moet worden ontworpen voor humane GvHD. Daarbij is tevens van belang dat er een goede samenwerking plaatsvindt tussen patholoog en kliniek, om tot een enkelvoudig graderingssysteem te komen voor humane GvHD in plaats van aparte systemen te houden voor patholoog en klinisch gebruik.

Rituximab behandeling in GvHD

Tot slot een woord over het gebruik van Rituximab in GvHD in de huidige praktijk, gebruik makend van de overwegingen die voortkomen uit onze analyse van B-cellen en Tregs als ook de bijkomende bevindingen met betrekking tot histologie versus klinische uitkomsten.

Zoals ook met huidbiopten, is timing essentieel als het gaat om B-cel depletie therapie met Rituximab. Timing van deze therapie is essentieel om GvHD symptomen te verminderen. We moeten ons wel realiseren dat verbetering een subjectief concept is in GvHD. Ondanks de klinische verbetering op Rituximab, kan dit middel immers niet voorkomen dat er op histologisch niveau toch schade optreed.

Alles in overweging nemend, zullen dokters dan ook moeten bepalen hoe men 'schade' wil definiëren in de eed van Hippocrates en moeten besluiten wat of wie men nu eigenlijk behandelt: behandelt men de symptomen van de patiënt of wil men daadwerkelijk voorkomen dat er vitale orgaanschade optreed? Hoe men 'schade' wenst te definiëren in de setting van GvHD na HCT zal bepalend zijn in hoeverre de dokter in staat is om zich te houden aan de eed hij/zij ooit gezworen heeft …

APPENDIX

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

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Zonder nog meer losse namen te noemen met het risico iemand te vergeten, maar zonder te vergeten hoe belangrijk jullie zijn: alle andere analisten en medewerkers van de pathologie in het UMCU. Jullie leerden me geduldig coupes te snijden (wie had ooit gedacht dat je een patholoog (in-wording) ook echt coupes kon leren snijden), te kleuren, RNA te isoleren en alles wat nodig was om dit voor elkaar te krijgen. Dank jullie wel voor jullie steun en oprechte interesse, ook toen ik al jaren in het oosten zat. Het voelde steeds weer als een beetje thuiskomen als ik in Utrecht kwam.

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Mijn familie en vrienden, ik besef volledig dat dit het enige hoofdstuk is dat niet medisch is of nadere uitleg vergt. Voor veel van jullie blijft mijn vakgebied toch een soort mysterieuze black-box en is de inhoud van dit boekje abracadabra (zelfs nu je het gelezen hebt), niettemin bleven jullie er altijd in geloven dat dit boekje zou slagen. Dank voor jullie continue ondersteuning gedurende al die tijd. In het bijzonder voor pap en mam: zonder jullie was dit nieuwsgierige meisje er niet geweest, laat staan dit proefschrift. Ik heb veel van jullie geleerd, vooral dat je nooit moet opgeven als het even tegenzit. Dank voor jullie steun en de mogelijkheid om altijd te gaan waar je hart je brengt.

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APPENDIX

CURRICULUM VITAE

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Marieke Cornelia Hendrika Hogenes was born on August 6th 1980 in Alphen aan den Rijn, the Netherlands. Her inborn interest in science was revealed as early as the age of only 4 years old, when she analyzed the hypothesis of potential snakes trapped in caterpillars in her parents back yard. After finishing her pre-university education at the Christelijk Lyceum in Alphen aan den Rijn, she started her medical training at the University of Utrecht in 1998.

At the anatomy science lab in her first year of medical training her attention was immediately drawn to pathology, when she discovered that diagnosing carcinomas in all their forms and presentations was one of the unique jobs of a pathologist: you could take a sample of tissue, have a look and make a diagnosis! In her 4th year of training she conducted her research internship at the Jordan Lab in the University Medical Centre Utrecht, adding in the analysis of a new xenogeneic mouse-model as *in vivo* model for graft-versus-host disease by histologically proving human cell presence within the murine organs, supervised by Dr. R.S. van Rijn and Dr. S. Ebeling. She won the first price for her poster presentation of her histological data at the Medical Students & Science day UMC Utrecht in 2003.

It did not surprise anyone when she decided to specialize in pathology after graduating medical school and she started her five-year specialist training at the same day as she got her medical degree (April 1th, 2005), at the University Medical Centre Utrecht with Professor Doctor J.G. van den Tweel. She never lost her interest in graft-versus-host disease though. During her last 5 months of traineeship in Utrecht, she initialized her study for this thesis.

Marieke completed her traineeship on April 1th 2010 and she's been working as a staff member at LABPON (Laboratorium Pathologie Oost-Nederland) in Hengelo ever since. She currently lives in Nijverdal, together with her husband Stephan, her 5-year-old daughter Eva and their four Maine Coons Istar, Mojo, Harmony and Hector.

APPENDIX

LIST OF PUBLICATIONS

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Publications included in this thesis

Hogenes M, Huibers M, Kroone C, de Weger R. Humanized mouse models in transplantation research. *Transplant Rev (Orlando).* 2014;28(3):103-10

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