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IMMUNOBIOLOGY OF GRAFT VERSUS HOST DISEASE IN CHEMOTHERAPY BASED CONDITIONING – *NEW MOUSE MODEL*

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To Scientists

Who are working anonymously

to make our life pleasant.

ABSTRACT

Introduction Graft versus host disease (GVHD) is a life threatening complication of allogeneic hematopoietic stem cell transplantation. The pathophysiology of GVHD is rather complex and several factors including; conditioning; MHC disparity and donor/host immune system influences both development and intensity of GVHD. Animal models have improved our knowledge about the different aspects of acute GVHD, however, these models should be closer to the clinical setting to increase the relevancy of the results.

Aims: The present thesis aimed to develop a new mouse model of acute GVHD based on chemotherapy conditioning to explore the immunobiological events in the pathogenesis of acute GVHD and to improve our knowledge about the underlying mechanisms in order to develop new treatment strategies and improve the clinical outcome.

Material and Methods: Female BALB/c mice were used as recipients while male C57BL/6 and female BALB/c were used as allogeneic and syngeneic donors. Busulfan (Bu) and cyclophosphamide (Cy) were used as conditioning agents. On the transplantation day, recipient mice were infused with either allogeneic or syngeneic bone marrow and spleen cells. GVHD was evaluated according to clinical manifestations, immunohistopathology and flow cytometry. Serum cytokines were measured using the Gyrolab Bioaffy technique and gene expression patterns in different tissues were examined utilizing the Affymetrix GeneChip technique.

Results: Similar treatment efficacy, engraftment and lower general toxicity were observed when the administration order of standard Bu-Cy was reversed to Cy-Bu.

The use of Bu (80mg/kg) for four days, followed by Cy (200mg/kg) for two days induced lethal GVHD in the BALB/c (H-$2k^{d}$) recipients transplanted with allogeneic graft from C57BL/6 (H-$2k^{b}$); mice receiving a syngeneic transplantation recovered. Furthermore, seven days post allogeneic BMT, donor T cells (mainly CD8+ T cells) migrated from the spleen to target tissues (skin, liver and intestine) initiating organ damage. In the early phase of GVHD, host DCs transiently expanded and initiated the inflammatory response however, donor DCs expansion and activation was more prominent than the recipients' DCs at early phase of GVHD (day+3).

Naïve T cells (CD3+$CD44^{low}CD62^{high}$) were primed by donor/host APCs in the spleen of the recipient to gain effector/memory cells (CD3+$CD44^{high}CD62^{low}$) phenotype. Subsequently, effector/memory cells migrated to peripheral tissues and induced tissue damage.

Gene expression analysis at day 0, showed that STAT1 and STAT3 were increased in liver while the antigen presentation, IL-4 and Leukocyte extravasation pathways were over expressed in the intestine. Gene expression pattern at day +7, in the allogeneic setting showed that antigen presentation, IFNγ, apoptosis, IL-1, IL-6 signaling and leukocyte trafficking pathways were mostly affected in the liver, intestine and kidney compared to syngeneic group.

Conclusions: These studies have shown that dose optimization may be used to improve the transplantation outcome. The newly introduced mouse model of GVHD have brought experimental animal models close to the clinical settings for further investigations of the mechanisms underlying GVHD as well as for evaluation of conditioning related toxicity.

We have also shown that naïve T cells undergo early allo-activation by host/donor DCs in the secondary lymphoid organs to produce effector/memory T cells that initiate tissue damage in GVHD. Prophylactic therapy should be used prior to donor T cell activation.

Gene expression analysis was shown to be vastly different in allogeneic compared to syngeneic transplanted mice and was synchronized with the clinical and biochemical changes. Gene expression analysis may be utilized for early diagnosis of GVHD.

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LIST OF PUBLICATIONS

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II. **Behnam Sadeghi**, Nasser Aghdami, Zuzana Hassan, Mehrdad Forouzanfar, Björn Rozell, Manuchehr Abedi-Valugerdi and Moustapha Hassan. GVHD after chemotherapy conditioning in allogeneic transplanted mice. Bone Marrow Transplantation (2008) 42, 807–818.

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CONTENTS

LIST OF ABBREVIATIONS

ALAT	Alanine aminotransferase
APC	Antigen presenting cell
ASAT	Aspartate aminotransferase
BM	Bone marrow
BMC	Bone marrow cell
BMT	Bone marrow transplantation
Bu	Busulfan
CD	Cluster of differentiation
CpG	Cytosine Guanine
CTL	Cytotoxic T lymphocyte
Cy	Cyclophosphamide
DCs	Dendritic cells
DLI	Donor lymphocyte infusion
FISH	Fluorescence in situ hybridization
GI	Gastrointestinal
GM-CSF	Granulocyte macrophage colony stimulating factor
GSH	Glutathione peroxidase
GVHD	Graft versus host disease
GVL	Graft versus leukemia
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
IFN	Interferon
IL	Interleukin
IP	Intraperitoneal
KIR	Killer cell Ig-like receptor
LPS	Lipo poly sacharide
mHag	minor histocompatibility antigen
MHC	Major histocompatibility complex
MoAB	Monoclonal antibody
MUD	Matched unrelated donor
NK	Natural killer
NS	Normal saline
PB	Peripheral blood
RIC	Reduced intensity conditioning
SP	Spleen
TBI	Total body irradiation
TGF-β	Transformin growth factor-beta
Th	T helper
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRT	Treatment related toxicity
VOD	Veno occulsive disease

1 INTRODUCTION

1.1 HEMATOPOIETIC STEM CELL TRANSPLANTATION

Hematopoietic stem cell transplantation (HSCT) has revolutionized our knowledge about stem cell biology and introduced a curative treatment for both malignant and non-malignant diseases as well as different metabolic disorders [1-5]. HSCT is an excellent example of transitional research that began with laboratory animal investigations and finally transferred into a successful clinical treatment [6-10]. The HSCT procedure consists of several steps including; elimination of recipient stem cells, eradication of tumor cells, suppression of the recipient immune system and finally, transplantation of stem cells from a suitable donor. HSCT is classified according to the antigenic divergence between donor and recipient as follows:

1. Autologous *(patient stem cells are harvested and re-infused after conditioning).*
2. Syngeneic *(donor cells are harvested from an identical twin sibling).*
3. Allogeneic *(donor cells are harvested from a relative or non relative source that may be different in some minor or major histocompatibility antigens).*

Regardless of the type of HSCT, pre-transplant treatment (conditioning) is mandatory [11]. Successful regimens should fulfill three main issues including; eradication of malignant (or dysfunctional stem cells), suppression of host immune system and provide a space for the new donor cells [11] (although nowadays there is an uncertainty about this requirement [12]). Total body irradiation (TBI) alone or in combination with cyclophosphamide (Cy) has been used in both experimental and clinical transplantation settings since the 1950's [6]. The first conditioning regimen introduced into the clinical HSCT was TBI [13]. Radiation is an excellent myeloablative treatment and is able to eliminate malignant cells in their "sanctuary" place. However, in order to increase the immunosuppressive effect, decrease radiation toxicity (by decreasing radiation dose) and to prevent graft rejection, cyclophosphamide (Cy) was added to the conditioning protocols [14]. Although the exact mechanism of action of radiation is not fully understood, it is suggested that cell death is mainly induced via free radicals that cause breakage of the DNA chain. Cy is an alkylating agent and a pro-drug, that was introduced into clinical practice in 1958 and is currently one of the most commonly used anticancer and immunosuppressive drugs [15]. Even though Cy

is known as an immunosuppressive drug, Fried *et al.* have shown that it may also diminish the number of bone marrow stem cells and progenitor cells via damage to the stromal supportive cells [16]. Several serious side effects of TBI have been reported such as higher risk of secondary malignancy [17], cataract [18] and growth retardation [19] especially in pediatric patients. As a result, an irradiation free conditioning regimen was introduced by Santos where he substituted TBI with busulfan (Bu) [20, 21]. Busulfan is an alkylating agent that mainly kills slow growing cells, hematopoietic stem cells, and causes marrow ablation [22, 23]. Bu and TBI have similar myeloablative properties however, higher engraftment level and a lower number of surviving CFUs in TBI treated mice compared to those treated with Bu have been reported [22]. This may indicate difference in their mechanism of action and toxicity. Despite being an effective myeloablative agent, Bu does not possess sufficient immunosuppressive effects [24]. Therefore, to achieve an ideal pre transplant conditioning regimen immunosuppressive drugs such as Cy should be added to the Bu conditioning protocol. Application of Bu-Cy conditioning in the clinical setting has disclosed proprietary side effects related to this conditioning e.g. veno-occlusive disease [25], hemorrhagic cystitis [26] and interstitial pneumonia [27]. Aside from regimen related toxicity almost all forms of conditionings produce general organ toxicities [28] that arise from non specific cytotoxic effects. The most important undesired and uncontrolled effect of conditioning is induction of apoptosis (or necrosis) in innocent (non-target) organs which initiates the inflammatory cascade [29]. Due to the unfavorable role of the inflammatory cascade in allogeneic HSCT, several attempts have been made to find treatment regimens with less inflammatory effects while keeping the same anticancer and lympho-myeloablative efficacy. These attempts resulted in non-myeloablative and reduced intensity conditioning (RIC) that was developed in experimental models and then applied in clinical practice [30, 31].

Another approach would be optimizing and individualizing the current conditioning regimen in order to achieve higher efficacy and minimize toxicity. This approach is more complicated due to several factors including; drug interactions, diagnosis, age, liver status and drug kinetics. Busulfan, as a main element in the conditioning regimen, is an active component which exerts its toxic effect on the malignant (and non-malignant) cells after entering the cells. Metabolism and detoxification of Bu occurs in the liver, mainly through conjugation with glutathione (GSH) [32, 33]. Metabolism of Bu by the hepatocytes depletes the GSH content of the liver up to 60% [34]. This depletion causes vulnerability of the liver and possibly the whole organism to toxic metabolites and oxidative stress. Bu was also reported to affect the liver's P450 enzymatic

complex, suggesting that the metabolism of other components using this pathway may also be altered [35]. In standard Bu-Cy conditioning, as introduced by Santos, Cy is administered after Bu [21]. Cyclophosphamide is a pro-drug which has to be activated by liver P450 enzymes [15]. Metabolism of Cy through the hepatic P450 enzymatic pathway produces active as well as toxic components e.g. 4-hydroxycyclophosphamide, choloracetaldehyde and acrolein [15]. As substantial levels of GSH are necessary to detoxify these metabolites it was hypothesized that the administration of Bu before Cy (conventional Bu-Cy regimen) may increase treatment related toxicity (TRT) due to the decreased GSH level in the liver and tissues (after four days of Bu). Given that Cy metabolites are the main components causing short term toxicity [36, 37]. The idea of reversing the administration order of Bu-Cy to Cy-Bu would therefore be of interest.

Experimental medicine, particularly animal models and especially HSCT models have vastly improved our knowledge concerning transplantation related complications and treatment related toxicity. Establishment and improvement of HSCT was primarily based on canine, mice and rat [9, 20, 38]. Several of these models are still valid and important in exploring different aspects of HSCT. Most existing GVHD animal models are based on radiation as a conditioning regimen. Despite the valuable knowledge obtained from radiation based GVHD models; due to the wide variety of different conditioning regimens, the different mechanisms of action and toxicity profiles among the used protocols; there is a great need for the development of new animal models based on chemotherapy conditioning.

1.2 GRAFT VERSUS HOST DISEASE

Currently, allogeneic HSCT is used world wide and many patients benefit from this impressive therapeutic approach. Similar to other medical techniques HSCT has drawbacks and complications. Among them is graft versus host disease (GVHD) which is the main hampering complication related to allogeneic HSCT. Clinical signs of GVHD have been reported since the first experimental [39] and clinical [5, 13] setting.

Graft versus host disease is classified as two distinct forms, acute and chronic based on the time of onset. Acute GVHD arises within the first 100 days after transplantation, and chronic GVHD occurs after day 100 post-transplantation [40]. Due to the differences in pathological features and the mechanisms of these two phenotypes [41], many researchers believe that the pathological classification of graft versus host disease is more accurate and relevant determinant of disease.

This is especially evident since acute GVHD can occur beyond 100 days (overlap syndrome) in patients transplanted using non myeloablative conditioning [42, 43].

Acute GVHD is manifested by damage to the skin, liver and the gastrointestinal tract mainly through Th1 pathway, whereas chronic GVHD is more similar to autoimmune syndromes (Th2 pathway), for example, scleroderma-like skin disease, salivary and lacrimal gland involvement (Sicca syndrome) [41].

The incidence of acute or chronic GVHD varies between different centers however, acute GVHD develops in 40% - 70% of all allogeneic transplanted patients [44] while chronic GVHD may occur in 28% - 100% of patients [44].

Billingham formulated [45] three basic elements for developing GVHD. First, the graft should contain immune competent cells, mostly donor T lymphocyte [46, 47]. Second; the recipient must be immuncompromised i.e. not able to reject transplanted cells and third, the recipient tissues must present antigens that are not expressed by the donor's cells [48]. Along with these fundamental requirements other variables play important roles in promotion and/or severity of GVHD e.g. intensity of conditioning [49], degree of major or minor histocompatibility disparity [50, 51], cytokine gene polymorphisms and killer immunoglobulin receptors (KIR) family of natural killer (NK) cells [52].

It is believed that GVHD is initiated by the conditioning regimen [53], however, the pathophysiology of GVHD is a complicated process with various contributing factors. The genetic background, immune status of host and donor and environmental factors play a role in the outcome and pattern of GVHD [54, 55]. However, to simplify the mechanisms underlying GVHD the consequences of events are summarized in three different but related phases [56] (Figure 1).

1) Tissue damage attributable to conditioning
2) Donor T cells activation
3) Immune based host tissue damage

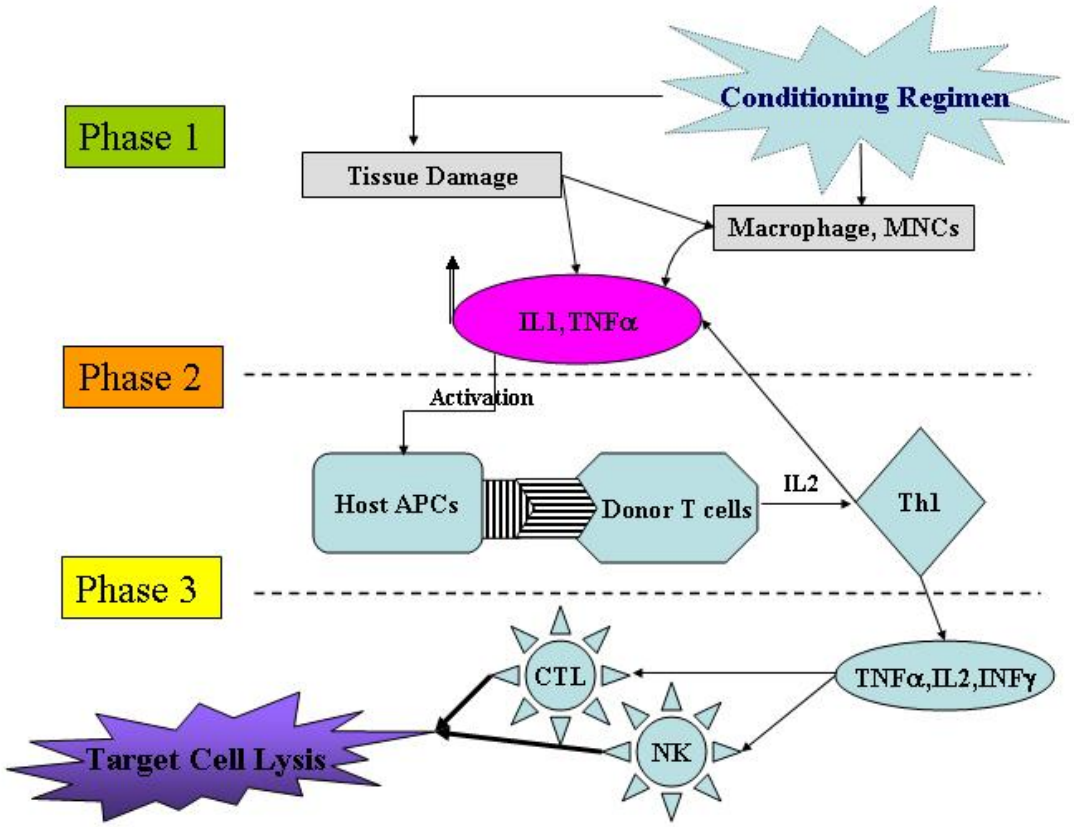

Figure 1. *Schematic map of GVHD*

In this hypothesis, the conditioning regimen induces tissue damage and release inflammatory cytokines. Following that, residual host (and donor) antigen presenting cells (APCs) capture the released antigens and differentiate into mature APCs. The activated APCs then present the host allo-antigens to the donor T cells which initiates the second phase of GVHD (donor T cell activation). Finally activated donor T cells migrate and invade different organs causing organ damage that is expressed as clinical manifestations of GVHD. In the following pages the risk factors and consequences of GVHD will be discussed in details.

1.2.1 The role of conditioning

Theoretically GVHD will develop after any allogeneic setting regardless of the type of pre-transplant conditioning [31]. GVHD can develop long after HSCT or even without conditioning as was observed in experimental models and clinical practice [47, 57, 58]. This indicates that the conditioning is an accelerator but not the exclusive triggering factor of GVHD.

Different forms of conditioning, either radiation, chemotherapy or both, are used prior to HSCT. The choice of regimen is dependent on the underlying disease, patient's age and availability of facilities [11, 28]. Any conditioning regimen, regardless of its intensity, induces tissue (especially GI) damage which leads to inflammatory cytokine release [59]. Also when the GI tract integrity is targeted by conditioning, lipopolysacharides (LPS) are released and translocate to the circulation [49]. LPS is a known potent immune stimulator that mediates inflammatory cytokine secretion [60]. Additionally, inflammatory cytokines will increase the permeability of the already damaged GI which results in an increased transmission of LPS into the circulation [61]. GVHD severity has been reported to correlate with the intensity of conditioning [53]. More intensive conditioning induces extensive tissue damage and higher levels of inflammatory cytokines which in turn induces severe GVHD [53]. The other aspect related to the conditioning, which should be considered, is the residual host immune cells which survive after conditioning. These residual cells have an important effect on the BMT outcome, occurrence and intensity of GVHD [62-64]. The balance between the host residual immune competent cells (e.g. NK, T cells) and incoming donor T cells determines the outcome of the HSCT [63, 64]. In other words, infusing alloreactive donor lymphocytes into an immunosuppressed recipient may shift the balance of attacking versus defending cells in favor of the donor alloreactive cells. This might be considered as the "Fighting theory". Under these conditions, donor T cells are dominant compare to host residual lymphocytes. Additionally, with less intensive conditioning, more host immune competent cells will survive which can deactivate or destroy some of the alloreactive donor T cells which results in an increment of graft failure [65] or delaying in GVHD or its severity. Gajewski *et al.* showed that addition of antithymocyte globulin to the pre-transplant conditioning regimen reduced graft failure by 50% [66]. In line with this hypothesis late donor lymphocyte infusion (DLI) convert mixed chimerism to full donor chimerism with a higher risk of GVHD and also GVL effect [58]. These findings indicate that changing the balance between donors versus host T cells and hence the outcome of HSCT may be achieved without changing the cytokine level.

1.2.2 **Contribution of genetic disparity**

The exclusive occurrence of GVHD in an allogeneic setting compared to a syngeneic or autologus transplantation setting, despite the similarity in conditioning regimen, implies the importance of antigenic disparity between donor and recipients in the pathogenesis of GVHD.

Human leukocyte antigens (HLA) in man or analogue molecules in animals has an exclusive function to draw an immune response either in physiologic, pathologic (autoimmune disease) or medical intervention (transplantation)[52]. Donor and recipients always differ in their genetic background (unless twins or syngeneic setting). This can be observed in the major or minor histocompatibility complex (MHC), Killer immunoglobulin-like receptors (KIR) [67] or cytokine genotype [68] which can influence the outcome of the graft. Differences in major or minor histocompatibility antigens between donor and recipient can results in allo-stimulation of donor lymphocytes and initiate GVHD [69]. However, various genes differ in their powers of eliciting strong allo-reactivity and GVHD [70]. HLA-A3 or C are associated with an increased risk of GVHD while DR1 is associated with a decreased risk [68]. The same phenomenon is applicable in class II MHC, while DRBI and DPBI have been associated with severe GVHD, a one allele mismatch in DP is tolerable [52]. These concepts imply the notion of permissible and non-permissible mismatch in the process of BMT.

Currently evaluation of HLA typing in A, B, C (class I), DRB and DQ (class II) with more precise molecular technique (DNA based HLA typing) is mandatory. However, serology based methods are still used in some centers.

To decrease the risk of GVHD the best combination of donors and recipients are twins or siblings who have similarities in both class I and II. This situation is called a full match and is available for 15%-30% of the patients [71]. However, even in HLA match siblings differences in minor histocompatibility antigens might play an important role in both GVHD and GVL effect.

The possibility of finding a fully matched unrelated donor (MUD) is about 30%-70% [71]. Due to the lack of suitable donors (or close relatives), haploidentical donors (some level of HLA incompatibility, either related or unrelated) will apply to 15%-50% of patients. The significance of MHC similarity and relationship between donor and recipients is demonstrated by the lower risk of mortality, [72] infection, [73] overall survival, [74] and the decreased risk of GVHD. Also, immune reconstitution occurs at an earlier time in matched and related donors compared to mismatched or MUD [75].

The occurrence of GVHD in HLA-matched settings is triggered by minor histocompatibility antigens (mHags) [52]. Minor histocompatibility antigens are peptides that are expressed by autosomal or Y chromosomes and presents either broadly or in limited tissues [76]. The first mHags that was shown to influence HSCT outcome was H-Y gene (Y chromosome) which was responsible in sex mismatched transplantation complications [77]. Some mHags are ubiquitously

expressed on all tissues (e g. H-Y, HA-3) whereas others (e.g. HA-1, HA-2) are limited to the hematopoietic system [78]. Different expression pattern of mHags in addition to various antigeneicity of the cytotoxic T cells is demonstrated by the severity of GVHD and GVL effect [52] which in turn outlines the role of the major or minor histocompatibility antigens in the pathogenesis of GVHD [78].

Some degree of MHC disparity is desirable, especially when HSCT is used in malignancies where strong GVL effect is warranted [79]. As mentioned above, the expression of some mHags are limited to hematopoietic system (like HA-1 and HA-2). Despite the fact that this disparity between donor and recipient may increase the risk of GVHD [78], it does however induce a strong GVL which is an important effect in the treatment of leukemia due to the expression on malignant stem cells that survive after conditioning.

Recently, Ringden *et al.* have shown that the GVL effect is lower or comparable in transplantations using unrelated donors compared to related donors [80]. In addition, Yakou *et al.* have reported that there is no significant difference in overall survival, GVHD and relapse among unrelated versus related matched donor providing selection of precisely matched unrelated donor [81].

In addition to the discrepancy in MHC molecules, polymorphisms of non-HLA genes like cytokines, cytokine receptors, KIR receptors and ligands may play an important role in promotion or prevention of GVHD [52, 82, 83]. Different alleles of cytokin genes (polymorphism) might affect cytokines both quantitively (expression level) or qualitatively (function) [84]. Middleton *et al.* have shown that recipients carrying tumor necrosis factor-alfa (TNF-α) genotype d3/d3 have a higher incidence of acute GVHD [82]. This observation was confirmed by the results reported by Cavet *et al.* [85]. In addition they have found a positive correlation between acute GVHD and early mortality rates in recipients with low production of IL-10 genotype (ACC) [85]. In contrast Nordlander *et al.* found no association between d3/d3 genotype of TNF-α and GVHD, however, they did find a relationship between GVHD and TNF-α d4/d4 [86]. Although several reports have shown the significance of host or donor non-HLA gene polymorphism in exacerbation or improvement of GVHD and/or transplantation related complications, the conclusions should be interpreted carefully because of the conflicting results. Taking account of all these reports, it would not be far from reality if in the near future, HSCT becomes an individualized treatment.

1.2.3 **Contribution of the immune system**

*1.2.3.1 **The role of cytokines***

Cytokines are considered as communication molecules in the immune system and play an important role in the pathogenesis of GVHD [87]. In fact, it has initially been proposed that the pathophysiology of acute GVHD can be considered as a "cytokine storm" phenomenon [88], which develops in three sequential steps. First, pretransplant conditioning regimen (chemotherapy and/or radiation) damages host tissues including the gastrointestinal tract, damaging the barrier which leads to the translocation of bacterial products (e.g., lipopolysaccharide (LPS) and CpG DNA) into the host circulation, promoting an inflammatory response in recipient body. This inflammation is characterized by the production of inflammatory cytokines such as TNF-α, interleukin (IL)-1 and IL-6 mainly from innate immune cells (monocytes, macrophages and dendritic cells (DCs)), which is the result of ligation of LPS with Toll like receptor 4 (TLR4) and CpG with TLR9 [89]. Second, as a consequence of increase in inflammatory cytokine production in the first step, the expressions of MHC molecules as well as other critical co-stimulatory molecules, which are required for the activation of allo-reactive T cells, are increased on both host and donor APCs. Consequently, donor T cells interact with these APCs, become activated by allogeneic major/minor MHC, undergo proliferation and differentiation, and finally produce abundant "T helper (Th) 1 type" or inflammatory cytokines (mainly TNF-α, interferon gamma (IFN-γ) and IL-2). Third, these cytokines in combination with other activated effector cells (e.g., NK cells and T cytotoxic cells) cause tissue damage. It is worth mentioning that the process of cytokine storm is concomitantly modulated by the generation of anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGF-β), which can be produced by both recipient and donor cells. Owing to the importance of pro- and anti-inflammatory cytokines in the development of GVHD, their functions are described in the following section.

TNF-α

Tumor necrosis factor-alfa is a typical pro-apoptotic cytokine and its role in the pathophysiology of GVHD has been studied extensively. TNF-α can be produced by many different cell types (76), but during GVHD is synthesized in large amounts by activated donor T cells and those of the monocyte/macrophage lineage. TNF signals through two TNF receptors, TNFRI (p55) and TNFRII (p75). These two receptors are typically coexpressed and can be found

on a variety of both hematopoietic and nonhematopoietic cells and signaling often results in the initiation of apoptosis [90]. Irradiation, ischemia/hypoxia, bacterial LPS, viruses, cytokines e.g. IL-1, IL-17 and IFN-γ can stimulate the production of TNF-α. Reciprocally TNF-α prompts the secretion of other cytokines like IL-1, IL-2 and IFNγ [91] implicating a central role of TNF-α in an inflammatory response and inflammation network. TNF-α induces apoptosis and necrosis in the inflamed tissue (locally) which lead to more inflammation and most probably more LPS leakage in the GI site [92]. Additionally TNF-α promotes the expansion of alloreactive T cells [93]. Interestingly, application of TNF-α neutralizing monoclonal antibody (MoAb) in clinical practice has been shown to improve the clinical manifestations of GVHD [94].

IL-1

The role of IL-1 as an inflammatory cytokine has been implicated in the initiation and promotion of GVHD (61, 68) and is produced by variety of immune and non immune cells (e.g., monocyte, macrophage, lymphocytes, NK cells, synovial fibroblasts, langerhans cells of the skin, mesangial cells of the kidney, vascular endothelial, thymic epithelial cells and smooth muscle cells) (66). During development of GVHD, IL-1 participates in a range of functions; it stimulates secretion of IL-1 (itself), IL-6, TNF-α and IL-12, granulocyte-colony stimulating factor (G-CSF) and TGF-β [61]. This cytokine also increases the vascular permeability as well as the expression of cytokine receptors for IL-2, c-kit and the common beta chain responsible for IL-3, IL-5 and granulocyte macrophage colony stimulating factor (GM-CSF) signaling [61]. Most importantly IL-1 activates T cells via either direct effect or increasing IL-2 secretion and IL-2 receptor expression on T cells [95]. IL-1 receptor antagonist (IL-1Ra) is the physiological antagonist to IL-1 molecule [96]. Mouse studies have shown that administration of exogenous IL-1Ra can improve the survival of GVHD mice [97, 98]. However, a clinical trial involving a large number of patients demonstrated that IL-1 blockade by exogenous IL-1Ra was not sufficient to prevent GVHD, possibly due to the dominance effect of TNF-α [99].

IL-6

IL-6 is an inflammatory cytokine, which is produced by T cells, macrophages, fibroblast and endothelial cells in response to tissue damage, infection and trauma [100, 101]. IL-6 is a pleiotropic inflammatory cytokine known to act on B and T cells, hepatocytes and hematopoietic progenitor cells [102]. Considering these biological functions, it has been shown that in patients with GVHD, prior to the development of clinical symptoms of the disease, serum IL-6 levels are increased [103]. In line with this finding, it has been demonstrated that the elevation in serum

levels of IL-6, IL-8 and TNF-α at the early phase after allo BMT (D 0-10) is associated with major transplant-related complications (eg. VOD, idiopathic pneumonia syndrome, GVHD grade II or higher) [104].

IFN-γ

IFN-γ is an inflammatory cytokine that is derived from activated T cells (Th1, Tc1), NK cells as well as activated APCs [105]. IFN-γ induces the secretion of IL-1 and TNF-α, and up regulates the expression of MHC molecules on immune and none immune cells, promotes the activation and differentiation of T cells and inhibits Th2 type of responses [106]. Moreover, it promotes maturation of APC via increasing the expression level of CD40 (which sends activation signals to DCs) [107]. It is now well established that in GVHD, IFN-γ plays a complex role with both pathogenic and protective effects [108].

It has been shown that IFN-γ is not necessary for the development of GVHD, using IFN-γ knockout mice as donor or recipients [109, 110]. Apparently it seems that high level of IFN-γ is protective against GVHD [111]. The reason for that could be the role of IFN-γ on homeostasis of activated T cells [112]. Unexpectedly, it seems that this cytokine protects the host from acute lung injury, but aggravates intestinal GVHD [108]. Beside the ambiguous effects of IFN-γ on the occurrence of GVHD, experimental models of GVL indicated a strong anti tumor effect of this cytokine in eradication of malignant cells [113]. The anti tumor effect of IFN-γ could be through direct toxic effect or indirectly by increasing the sensitivity of malignant cells to cytotoxic T lymphocyte (CTL) [114]. These data provoke some hope to separate GVHD from GVL effects by administrating IFN-γ at different time points and by targeting different cytotoxic T cells populations.

IL-2

IL-2 has long been considered to be the primary cytokine involved in the pathogenesis of GVHD. IL-2 is produced exclusively by activated T cells and promotes proliferation and differentiation of T, NK and B cells [115]. Biological function of IL-2 is multi-directed, consisting of both activation and suppression of immune functions [115]. IL-2 increases its own secretion and through expression of IL-2Rα (CD25) and IL-2Rγ promotes T cell survival and differentiation to effector and memory cells, and acts as a autocrine growth factor [116]. IL-2 might regulate immune responses either through optimizing central/peripheral Treg cells survival and function [117] or via inducing T cell apoptosis through activation-induced cell death (AICD) [118]. Regarding this issue, initial studies have shown that administration of exogenous IL-2

intensifies the GVHD manifestations [119]. In contrast, later investigations demonstrated that early *in vivo* administration of IL-2 with T cell-depleted BM, permits alloengraftment and prevents GVHD [120]. In a later report from the same lab, it was shown that IL-2 administration inhibit the GVHD-producing activity of allogeneic CD4+ T cells, but preserve the GVL effects of allogeneic CD8+ T cells [121]. It seems that in both reports, the protective effects of IL-2 administration arises from the induction of Treg (CD4+CD25+FoxP3+ cells) cells population and/or Th17 function by IL-2 [122-124]. Recent advances in the understanding of IL-2 function have lead to a new biological therapy for the GVHD. IL-2 exerts its function through binding to a transmembrane IL-2Rα receptor (CD25), so Basiliximab and Daclizumab are two chimeric monoclonal Abs that are designed to prevent IL-2 function *in vivo* [125, 126]. Several reports in recent years have shown that the administration of blocking antibody might reduce the intensity or prevent GVHD even refractory form [127, 128]. Considering these paradoxical observations and based on the fact that IL-2 possesses multifaceted biological functions, it seems that the doses of IL-2 and administration time points (either IL-2 or blocking Abs) are vital factors that influence GVHD outcome. More exploratory work is necessary to clarify these issues.

IL-17/IL-23

Interleukin 17 is a recently introduced pro inflammatory cytokine that has gained an enormous interest in relation to both autoimmune diseases and inflammatory reactions [129]. The IL-17 family consists of six members in which IL-17 (IL-17A) and IL-17F have high similarity in structure and biological function [129]. IL-17 exerts its function through IL-17 receptor that is ubiquitously expressed in tissues [130]. Although, IL-17 is mainly produced by a distinct lineage of CD4 cells (Th17) [131], other immune cells such as CD8+ T, NKT and γδT cells, and neutrophilic granulocytes can secrete IL-17 under certain conditions [129, 132]. It has been shown that TGF-β in combination with IL-6 and IL-1β strongly promote T cell trans-differentiation to the Th17 lineage [133]. In this context, it seems likely that Treg cells subvert Th1 and Th2 differentiation for the generation of IL-17-producing T cells [134]. Data also suggested that Th1 and Th17 responses are reciprocally regulated i.e., Th17 differentiation is suppressed by the Th1 cytokine IFN-γ [135]. In connection with this issue it has been shown that in the absence of donor-derived IL-17, a potentiated Th1 profile develops, which leads to an increase in GVHD mortality [136]. Undoubtedly, more studies should be performed to fully address the role of Th17 cells and their related cytokines in the modulation of GVHD process.

IL-4

Interleukin-4 is mainly produced by activated helper CD4+ cells, activated mast cells and basophiles. Interleukin-4 (IL-4) is crucial for the differentiation of naive T helper cells into the Th2 effector cells that promote humoral antibody responses and provide protection against intestinal helminthes [137]. IL-4 also has a central role in the pathogenesis of allergic inflammation [138]. Based on the fact that IL-4 antagonizes the biological and immunulogical activities of IFN-γ [139], it has been suggested that IL-4 might act as a modulatory factor of acute GVHD [140, 141]. However, this suggestion did not find further support, since absence of IL-4 in donor cells exert a protective rather than a deleterious effect on the development of GVHD [110]. Thus, owing to the complex network of Th1, Th2 and Th17 cytokines involved in the pathogenesis of GVHD, more comprehensive studies are required to fully understand the role of cytokine in the development of GVHD.

IL-10 and TGF-β

Interleukin-10 (produced mainly by B cells, Treg cells and DCs) and TGF-β (produced platelets, myeloid and Treg cells) are two cytokines with wide-ranging effects on immune cellular processes [142, 143]. They can suppress both Th1 and Th2 pathways and modulate inflammatory responses. Based on their immunosuppressive properties, IL-10 and TGF-β can be considered as proper therapeutic candidates for the treatment of GVHD. Regarding the effects of IL-10 on GVHD, Baker *et al.* have shown that higher IL-10 production by recipients (before BMT) is associated with less transplant related complications including GVHD and death [144]. On the other hand, Hempel *et al.* have reported that high IL-10 level after allo BMT is accompanying with higher mortality and more severe complications [145]. Thus, depending on administrated dose and time IL-10 might have dual and opposite effects, either promote or prevent, GVHD [146, 147].

In the case of TGF-β, the results from several *in vivo* and *in vitro* studies have indicated that this cytokine plays a specific role in inhibition of T-cell responses to alloantigens [148, 149]. In connection with this issue, experimental studies have implied that TGF-β predominantly produced by donor T cells immediately after transplantation attenuates the development of acute GVHD [150]. Conversely, the same cytokine produced by myeloid cells long after the transplantation promotes chronic GVHD [150]. The existence of this paradox can be explained by the possibility that during the early acute inflammation, the immunosuppressive effects of TGF-β are dominant, whereas, later this cytokines play a pivotal role in induction of fibrosis,

which is a characteristic of chronic GVHD. Again, much research work should still be performed to understand the timing and location of TGF-β production, the role of other cytokines that complement or antagonize the actions of this cytokines with regard to the development of GVHD.

1.2.3.2 **The role of immune cells**

In general, GVHD resembles a normal immune response [151], i.e., the disease is activated when the grafted donor T lymphocytes recognize the host as foreign (Non self) [55, 152, 153]. Thus, APCs and donor T cells are the most important cells that participate in the course of GVHD. On the other hand other immune cells such as Treg, B and NK cells might also be involved in the modulation of the disease. Due to their importance, these cells are separately discussed in the following section.

Dendritic (DC) cells

A proper activation of APCs is necessary for stimulation of donor T-cells. As mentioned previously, pre-transplant conditioning induces damage to malignant and non-malignant cells as well as highly proliferative epithelial tissues [53]. Subsequently, systemic exposure to microbial products such as lipopolysaccharide (LPS) intensify inflammatory cytokines secretion (e.g. IL-1, IL-6 and TNF-α) that stimulate APCs [56]. Experimental data have demonstrated that both host and donor antigen presenting cells participate in the pathophysiology of GVHD [153-155]. Using a minor histocompatibility-antigen model, Shlomchik *et al.* and Teshima *et al.* have demonstrated that, host APCs present allo-antigens to donor CD8+ or CD4+ cells [62, 152]. On the other hand, several reports have shown that donor-derived APCs are also able to promote GVHD, by acquiring and presenting host antigens (cross-priming) [48, 55, 153, 155]. These studies suggest that DCs will present host antigens (minor/major histocompatibility complex) to donor T cells. Thus DCs can olay a crucial initiator as well as promoter of GVHD.

Recent published results imply an inhibitory function for DCs in the pathogenesis of GVHD [156, 157]. In this respect the role of other antigen presenting cells such as monocytes/macrophages or B cells may be considered. Current data suggest that host B cells may reduce acute GVHD under certain condition [158]. Additionally, it was shown that host or donor mesenchymal stem cells, may also reduce the incidence of acute GVHD, although the mechanisms remain to be explored [48, 159]. DC activation begins by encountering with an

antigen [151]. Antigens/pathogens stimulate the immature DCs and induce phenotypic and functional changes in these APCs. Upon stimulation, DCs are converted from Ag-capturing cells (immature) to Ag-presenting (mature) cells. After maturation, DCs migrate from the peripheral tissues to the draining lymphoid organs [151, 160], where in the T-cell dependent area, they present the antigen-derived peptides in the context of MHC I [161] or MHC II to the CD8+ or CD4+ T lymphocytes, respectively [151, 160]. Expression of antigens to naïve T lymphocyte should be accompanied with the expression of other co-stimulatory molecules like CD80 and CD86 on DCs [162].

T cells may also activate DCs via CD40L-CD40 signaling pathway and secretion of cytokines like IL-1, IL-2 that increase CD80/86 expression on DCs [163]. CD40L is a strong stimulatory molecule for DCs and is expressed on activated CD4+ cells [164, 165]. During the inflammatory process, expression of CD40L is increased, which leads to a strong interaction between this molecule and its cognate receptor, CD40 molecule on DCs, and thereby promotes DCs maturation and increases CD80/86 expression on these cells [164, 165].

T cells

T lymphocytes are crucial player in adoptive immunity. According to the fate of immune response and cytokine milieu, after activation and proliferation, they can differentiate to Th1, Th2, Th17 and/or T regulatory cells. Th1 pathway with its particular cytokines (TNF-α, IL-2 and INF-γ) is associated with acute GVHD [166]. Impairment in the production of Th1 cytokines might either dampen or deviate the pattern of acute GVHD through increasing the number of Treg cells or enhancing other inhibitory/compensatory (e.g., Th17 cells) mechanisms [167-169]. Regarding this issue, it has also been suggested that polarization from Th1 to Th2 type of response, suppresses the acute GVHD, but increases the risk for the development of chronic GVHD [166, 170]. However, other studies have shown that cross talk between these pathway might be involved in both acute and chronic GVHD [171].

Although, the role of T lymphocytes in the pathogenesis of acute GVHD has been well established [46, 47], attempts to deplete T cells in the graft in order to prevent GVHD, have resulted in increased relapse rate and graft failure [172-174].

The co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) expressed on APCs regulate T cell responses through activating signals via CD28 and/or inhibitory signals through CTLA4 that are expressed on naïve and activated T cells [175]. Several reports have emphasized the

importance of B7:CD28/CTLA4 pathways on the control of GVHD [176-178]. In this respect, it has been demonstrated that blocking the B7:CD28 pathway is more effective in preventing of GVHD compared to stimulating B7:CTLA4 pathway [175, 178].

Depending on the quantity and quality of peptide-MHC ligands, T cell activation will be completed between 24 hours to 3-4 days [179]. In a mouse model of acute GVHD, we have shown that T cell activation occurs between 3 to 5 days after graft infusion (Sadeghi *et al.* /paper 3). Moreover, Beilhack *et al.* have shown the same time interval for the proliferation of T cells in secondary lymphoid organ [180]. During the activation phase, donor T cells also secrete cytokines [163] or express co-stimulatory molecules to enhance DCs function to improve activation efficacy [164, 165].

It has been shown that disparity in MHC class I can cause lethal acute GVHD, which is governed by CD8+ T cells, whereas differences in MHC class II account for acute GVHD pathology, which is induced by CD4+ T cells [62, 152, 181]. However, experimental data have shown that even in CD8+ mediated GVHD, small population of CD4+ cells in the graft accelerate GVHD lethality and vice versa [182]. In a well-designed study, Berger *et al.* have shown the reciprocal synergistic effects of CD4+ and CD8+ T cells in enhancing the severity of GVHD [183]. These results indicate the complexity of immune network and reflect the possible roles of other immune components in boosting immune response. In other words, we might be able to dissociate CD4+ or CD8+ T cells function in genetically manipulated animals however, this is not possible in clinical practice due to the complexity and integrity of the immune components which control for the final outcome.

Although activation of donor T cells mediate GVHD as a life threatening complications of HSCT, donor T cells are necessary for both successful stem cell engraftment [172, 173] and graft versus leukemia (GVL) [174] effect. GVL is an important part of biological anticancer therapy especially in the RIC or non-myeloablative conditioning regimens. Experimental and clinical data indicate that CD8+ cells are important to prevent graft failure and promote GVHD while both CD4+ and CD8+ cells have pivotal roles in GVL (157, 158).

Another important aspect of GVHD immunopathology is the potential role of effector memory T cells in the pathogenesis of GVHD. Theoretically, only naïve T cells ($CD44^{low}CD62^{high}$) could be primed by activated DCs in the process of inflammatory situation like GVHD. However, cross reactivity with similar allo-antigens among some clones of effector memory cells should be kept in mind. This issue is more prominent especially in the human with

higher possibility of previous antigenic encountering, compared to isolated and pathogen free experimental animals. Regarding this issue, the results from several studies have shown that activated naïve T cells initiate tissue damage in acute GVHD [184, 185]. On the other hand, other studies have demonstrated that effector/memory T cells ($CD44^{high}CD62^{low}$) contribute to either the initiation of GVHD [186, 187] or to the induction of late onset of GVHD with low severity [188]. Together, activated donor T cells migrate to various tissues, colonize and infiltrate target organs (skin, intestine, lung and liver are mostly reported) and induce tissue damage via cellular or cytokine mediated immune response [189].

T regulatory (Treg) cells

Regulation of immune response is an essential mechanism to control inflammation. Treg cells, characterized by $CD4+CD25^{high}FoxP3+$, are among the most important regulatory cells in the body [190]. These cells constitute 1%-5% and 5%-10% of CD4+ T cell population in mice and human, respectively [191]. In addition to Treg cells, other innate or adoptive immune cells e.g., CD8+, CD8+CD28- and NKT cells can also exert regulatory effects on immune mediated responses [190]. Together, these cells form a regulatory network that can control inflammation and prevent autoimmune reactions in pathological and physiological situation via cellular or cytokine mediated mechanisms.

Treg cells mediate their regulatory effects via secreting IL-10, TGF-β or via direct cell to cell contact [192, 193]. As mentioned previously, IL-10 and TGF-β inhibit the secretion of pro-inflammatory mediators e.g. IL-1, IL-6, IL-12, TNF-α and IL-2 produced by the activated immune cells (e.g., monocyte, macrophage, DCs, T and NK cells) [194]. Treg cells can also suppress the activation of T cells through down regulation of co-stimulatory molecules (CD80/CD86) on the surface of DCs [195]. Additionally, Treg cells might mediate their inhibitory effects via killing effector T cells using the FAS-FAS ligand pathway [196].

Several reports have illustrated a protective effect of natural (freshly isolated) or *ex vivo* expanded Treg cells on GVHD [192, 197]. These data indicate that co-transfer of Treg cells with the graft might prevent or at least decrease the intensity of GVHD. However, the feasibility of employing Treg cells in clinical settings is hampered by the lack of proper procedure to isolate high numbers of Treg cells which is required to efficiently prevent GVHD [193, 198]. Moreover, the results from a study have shown that in contrast to experimental models, patients with donor graft containing high numbers of CD4+ CD25+ cells develop more severe GVHD [199]. The

authors suggested that in human, co-expression of CD4+ and CD25+ on donor cells is not sufficient to induce an immune regulatory function. In this respect, a recent report by Ermann *et al.* implied that only Treg cells expressing CD62L+ are able to prevent donor T cell expansion and consequently protect the host from lethal GVHD [200]. They concluded that the ability of Treg cells entering to the secondary lymphoid organs is critical for their inhibitory function [200]. It is worth mentioning that the ultimate goal for the adoptive transfer of Treg cells is to suppress GVHD while preserving the GVL effects [201].

Natural killer (NK), Natural killer T (NKT) and B cells

Natural killer cells or large granular lymphocyte are one of the major cell components of the innate immune system and constitute about 10% to 15% of circulatory lymphocytes [202]. These cells neither express T nor B cell recprtor markers. NK cells do not require to be activated by APCs in order to kill cells that are missing "self" markers of MHC calls I [203]. However, presence or absence of MHC class I is not always inhibitory or permissive of NK cell function [203].

The importance of NK cells in the field of BMT was first explored by the ability of host NK cells to reject donor grafts [204]. Pretreatment of the host with anti-NK antibodies decreases the intensity of conditioning required for engraftment [205]. NK cells do not have a direct role in initiation of GVHD. However, in the last phase of GVHD in which donor T cells release inflammatory cytokines and recruit other cytotoxic cells to the target tissues, NK cells mediate tissue damage. On the other hand several reports have shown that NK cells can prevent or decrease the intensity of GVHD while maintaining the GVL effect [203, 206, 207]. This function could be explained by the MHC class I differences between the donor and host. In this case, donor NK cells recognize and eliminate residual host DCs (APCs), which results in the reduction of GVHD [62].

NKT cells are are a heterogeneous group of lymphocytes that share properties of both T cells and natural killer (NK) cells. They co-express an αβ T cell receptor and many of these cells recognize the non-polymorphic CD1d molecule, an antigen-presenting molecule that binds self and foreign lipids and glycolipids [202, 203]. They constitute only 0.2% of all peripheral blood [202, 203]. Upon activation, NKT cells can either stimulate or inhibite T cell activation via secretion of IFN-γ and TNF-α or IL-4, respectively [208]. Several reports have shown the essential regulatory function of host NKT cells in the prevention of GVHD through the secretion

of regulatory cytokines such as IL-4, IL-10 or TGF-β [64, 207, 209]. In line with this finding, Pillai *et al.* have shown that while host NKT cells prevent GVHD via inhibiting donor T cells activation, they permit GVL activity of donor cytotoxic cells [210]. Moreover Margalit *et al.* have shown that adoptive transfer of donor NKT cells improve allo BMT outcome and decrease GVHD tissue damage and mortality rate in a dose dependent manner [211]. They have found that Th2 cytokines (IL-4 and IL-10) was increased in the serum of recipients of NKT cells [211]. Thus, although it should be experimentally proven further, NKT cells might have a clinical potential to prevent or treat GVHD.

B lymphocytes are in the main component of the humoral immune system and are closely related to Th2 pathway. However, they are also considered as semiprofessional antigen presenting cells since they can present antigens to T cells through surface immunoglobulins [212]. Due to the sensitivity of B lymphopoiesis to conditioning [213] and profound impairment of these population during GVHD, [213, 214] their role in the pathogenesis of acute GVHD is not completely understood. However, few studies have pointed out the role for B cells in promotion and development of chronic GVHD through inducing Th2 pathway [215, 216].
Considering the traditional function of B cells in presenting antigens to T lymphocytes, it seems that B cells might aggravate GVHD. Nevertheless, recent publication by Rowe *et al.* demonstrated opposite results [158]. In a MHC mismatched mouse model of GVHD, they found that B cell deficient recipients developed more severe GVHD compared to wild type host. They have also shown that the protective effect of host B cells is mediated by secretion of IL-10 and increased level of IFN-γ in recipients. In contrast, Schultz *et al.* have shown that host B cells can increase the severity of GVHD [217]. The contradictory results obtained by these two studies might be due to strain disparity. The GVHD model in Schultz study consisted of a MHC matched minor Ags mismatched while, Rowe *et al.* used MHC mismatched strains [158, 217]. To explore the role of host APCs in promotion of GVHD, Duffner and collageues added back wild type DCs or B cells to the MHC class II deficient recipients [154]. This procedure demnstrated that only DCs of host origin are able to induce GVHD, via interaction with donor T cells, whereas host B cells were inactive [154].

Th-17 cells are recently discovered T cells with strong inflammatory function [218]. Their main role is eradicating pathogens that are not responding to Th1 or Th2 cells [218]. As their

name suggest, Th-17 cells produce mainly IL-17 family (six member, IL-17A to IL-17F), IL-22 and IL-23 [218]. Generation of Th-17 cells from naïve T cells is mainly under the control of TGF-β and IL-6 [218]. Th-17 cells act opposite to Treg cells and mediate strong inflammatory milieu. Principally, development of Th-17 and Treg cells are reciprocally controlled by TGF-β and IL-6 [218]. TGF-β induces development of Treg cells in the absence of IL-6. However, in the inflammatory situation, increased expression of IL-6 in combination with TGF-β abrogate Treg formation and instead shift T cells to generate Th-17 cells [218].

Despite that the role and contribution of Th-17 cells in the pathogenesis of GVHD is still unclear, most of the recent reports indicate a critical role for donor Th17 and its cytokine (IL-17) in the promotion of acute GVHD [136, 219-221]. Lack of Treg cell function [221] and/or compensatory hyper activation of Th1 pathway [136] were discussed as possible reasons for exacerbating of GVHD. In line with these findings and due to probable mutual effects between Th-1 (IFN-γ) and Th-17 (IL-17), the development of GVHD in the absence of donor IFN-γ secreting cells might be explained [109, 136, 220]. Furthermore, it is still not clear whether Th-17 pathway is preceding Th-1 pathway in the process of inflammation or vice versa [222].

1.2.4 **Tissue damage – Final phase of GVHD**

During the last phase of GVHD, activated donor T cells migrate to peripheral tissues and mediate tissue destruction [48, 213, 223]. Moreover, in this process inflammatory cytokines and chemokines, those are produced by activated T cells and affected organs, recruit NK, macrophage and other cytotoxic cells to the inflamed area and aggravate the damage [54, 224]. Tissue damage is a function of cell mediated or cytokine toxicity [55]. Priority and importance of these pathways depends to the tissues and type of activated T cells which is determined by class of MHC disparity. The common belief is that Th1 mediates acute GVHD (through secretion of IFN-γ, IL-2 and TNF-α) while Th2 reduce severity of acute GVHD and preferentially promote chronic GVHD (via secreting of IL-4, IL-10, IL-13 and TGF-β) [225-227]. However, using STAT4 nulls (in which Th1 is absent while Th2 is active) and STAT6 nulls (in which Th2 is absent while Th1 is active) as donors, Nikolic *et al.* have shown that acute GVHD develops through both Th1 and Th2 however, mortality of acute GVHD and weight loss was more severe in the recipients of intact Th1 compared to Th2 [171]. Interestingly, histopathological evaluation of the recipient mice have shown that intestinal damage was mediated by either Th1 or Th2 whereas hepatic and skin damage was essentially induced by Th2 pathway [171]. This study

confirmed the complicated pathogenesis of acute GVHD. Furthermore it indicates that the Th1 and Th2 cells have various tropism for different target tissue [171]. Moreover, preferences of these pathways are strongly dependent on level, number and location of MHC disparity between donor and recipient.

Effector donor cells including CD8+, CD4+ and NK cells induce tissue damage via direct contact (FAS-FAS ligand), TNF (apoptosis via TNF receptor signalling) or through secretion of perforin/granzyme lymphokines [228-230]. On the other hand, TNF-α and IFN-γ that are Th-1 inflammatory cytokines and secreted by variety of inflammatory cells, mediate cell apoptosis through TNFR [48].

Experimental data have confirmed different tissues have various sensitivity to cytokine mediated toxicity [231]. Using TNF-α blocking antibodies Pigute *et al.* have shown that skin and intestinal lesions were more less severe as compared to control mice [232].

Baker *et al.* have shown that allo-transplantation of FAS ligand deficient T cells induced less hepatic GVHD which might indicate that FAS mediated cell toxicity is important for liver acute GVHD [233]. On the contrary, Tsukada *et al.* [234] could not observe such protective effect on the liver. In agreement with Baker *et al.*, van Den Brink *et al.* have shown that absence of FAS receptor on liver cells (FAS deficient recipients) prevent liver GVHD, which confirm the pivotal role for FAS-FAS ligand pathway in liver acute GVHD [235]. Burman *et al.* have shown that IFN-γ mediate GI damage however, it prevents the induction of idiopathic pneumonia syndrome during the progress of GVHD [108].

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Altogether, experimental and clinical data emphasize that the MHC products dictate the allo-immune response and consequently the pathogenesis of GVHD. Since MHC proteins are expressed on almost all nucleated cells (at least class I and some minor MHC molecules), the main question that arises is why some particular tissues (GI, liver, lung and skin) are the main targets in acute GVHD? The answer to this question will provide a way to develop therapeutic protocols and expand application of HSCT to a wider range of diseases.

2 GENERAL AIM OF THE THESIS

The present studies aimed to increase the knowledge about the pathophysiology of graft versus host disease.

Specific aims

- To evaluate the efficacy and toxicity of busulfan–cyclophosphamide (Bu-Cy) versus cyclophophamide–busulfan (Cy-Bu) conditioning in a syngeneic transplantation mouse model.
- To develop and establish mouse model of bone marrow transplantation and acute GVHD based on chemotherapy conditioning.
- To explore the immunopathology of early events in the pathogenesis of acute GVHD.
- To investigate the gene expression pattern among target and non target tissues after chemotherapy conditioning.
- To Study the gene expression profile at the beginning of acute GVHD in target and non target organs.

3 METHODS AND MATERIALS

3.1 ANIMALS

In all of the following studies two strains of mice; BALB/c (H-2Kd) and C57BL/6 (H-2Kb) were included. According to the protocols mice were considered as donor or recipients. Mice aged between 8-12 weeks at the time of experiments. Animals were housed in individual ventilated cages and maintained under specific pathogen-free conditions in an animal facility with controlled humidity (55% ± 5%), 12 hours light/dark, temperature (21°C ± 2°C) and HEPA filtered air. Animals were fed with autoclaved mouse chow and tap water *ad libitum* and were allowed to acclimatize to their surrounding for one week before experiments. All animal experiments were approved by the southern ethical committee and in accordance with the Animal Protection Law, the Animal Protection Regulation and the regulation of the Swedish National Board for Laboratory Animals.

3.2 CHEMICALS

L-α-phosphatidylcholine and 1,2-dioleolyl-sn-glycero-3-phosphate were purchased from Polar Lipids, Alabaster, Canada. Cholesterol (cholest-5-en-3β-ol), busulfan, cyclophosphamide were obtained from Sigma, Germany. RPMI1640 medium, phosphate-buffered saline (PBS), trypan blue and Iscove's modified Dulbecco's medium and fetal bovina serum (FBS) were obtained from Invitrogen, Stockholm, Sweden. Stain Buffer (FBS), anibodies and Perm/Wash were supplied by BD Biosciences, Pharmingen, San Jose, CA, USA. Several antibodies were purchased from eBiosciences, San Diego, CA, USA).

3.3 PREPARATION OF LIPOSOMAL BUSULFAN

Liposomal Bu was used for the first study and was prepared as described previously [236]. Briefly L-α-phosphatidylcholine, 1,2-dioleolyl-sn-glycero-3-phosphate, and cholesterol were weighted and dissolved in chloroform. Busulfan (Bu) was dissolved in dichloromethane and added to the mixture of lipids. The mixture was dried until a thin film was formed by evaporation. Glucose (50 mg/ml) was added and the mixture was filtered under nitrogen pressure utilizing extruder (LiposoFast 50, Avestin, Ottawa, Canada). Two polycarbonate filters (pore size 100 nm) were used. Liposomal Bu was stored at 4°C until use. Liposomal Bu concentration was

measured using gas chromatography with electron capture detection, as described previously [237].

Cyclophosphamide (Cy) was dissolved in sterile water, diluted to the final concentration and stored at 4°C prior to use.

3.4 CONDITIONING

Except for the first paper where liposomal busulfan and reversed administration order of the Bu-Cy were used (paper I), in all following studies recipient mice underwent Bu-Cy conditioning for 6 days (day -7 to -2) before the transplantation.

Recipient mice received 80-100 mg/mL Bu dissolved in DMSO at the concentration of 40mg/mL in four consecutive days (from day -7 to -4 before transplantation). Cyclophosphamide dissolved in sterile water at a concentration of 10 mg/mL. Each recipient received a dose of either 200 or 300 mg/kg at days -3 and -2. Days -1 and 0 were considered as resting and BMT days, respectively. Control mice received vehicles (DMSO and normal saline) based on the same schedule as treatment groups. All drug administrations were carried out in I.P route.

3.5 BONE MARROW TRANSPLANTATION

In all experiments bone marrow transplantation was carried out at day 0. Donor mice (either allogeneic or syngeneic setting) were killed by cervical dislocation. Bone marrow cells (BMC) were flushed from both femurs and tibias. Single-cell suspension was prepared by passing cells gently through a 14G needle in RPMI-1640 containing 2% FBS. Cells were then centrifuged and washed with RPMI. Donor spleens were disrupted in RPMI-containing 2% FBS to form single cell suspension. Splenocytes were passed through a 70 μm strainer, centrifuged and washed two times with RPMI. Cell number and viability was measured using Trypan blue exclusion dye.

Recipient mice received 20×10^6 BMC in combination with 30×10^6 splenocytes through lateral tail vein. Except in the paper I in which recipient mice received 6×10^5 Sca-1+ cells.

3.6 ASSESSMENT OF GVHD

Recipient mice were monitored daily starting from conditioning start until the appropriate sampling day for the clinical manifestations of GVHD (e.g., weight loss, hunched posture, poor activity, ruffled fur and loss of skin integrity) [141] (Table 1). The severity of each given symptom was scored from 0 to 2. The sum of the scores for all symptoms in each mouse

(maximally 10) was used as an index of the severity and progression of GVHD. Each experiment was repeated 4-5 independent times.

Table 1. Assessment of GVHD in mice

Criteria	Grade 0	Grade 1	Grade 2
Weight loss	<10%	>10% to <25%	>25%
Posture	Normal	Hunching noted only at rest	Severe Hunching impairs movement
Activity	Normal	Mild to moderately decreased	Stationary unless stimulated
Fur texture	Normal	Mild to moderate ruffling	Severe ruffling/poor grooming
Skin integrity	Normal	Scaling of paws/tail	Obvious areas of denuded skin

3.7 SINGLE CELL PREPARATION AND FLOW CYTOMETRY

a. Bone marrow and spleen

At appropriate time points, recipient mice were killed, spleens and femurs were removed. A single cell suspension of splenocytes and BMC's were prepared as described in paper II. Erythrocytes were lysed with ammonium chloride. The obtained single-cell suspensions were washed twice in PBS and re-suspended in FACS buffer solution.

b. Isolation of epidermal immune cells

Epidermal immune cells in the skin were isolated as described previously [69]. Briefly, pieces of skin (abdomen) were placed in a micro-tube containing 1ml Dispase and incubated overnight at 4°C. Epidermis was removed and the tissues were cut into small pieces. Samples were then digested in Dulbecco's modified Eagle's medium containing HEPES, DNase, collagenase D, and hyaluronidase at 37°C for 1.5 hr. Thereafter, the digested tissues were filtered through a 70µm strainer to obtain single-cell suspension.

c. Isolation of intestine intraepithelial lymphocytes

Intestinal lymphocytes (intraepithelial) were isolated as described elsewhere [238]. Briefly, small intestine was dissected and the lumen washed with cold PBS-2%FBS. The intestine was cut and placed in an Erlenmeyer flask containing PBS-FBS-EDTA. The flask was incubated for

20min at 37^{o}C under stirring. After 15sec vigorous vortex, the supernatant was filtered and collected.

d. Immunofleurocens staining of cells using FACS

Flourochrome-conjugated monoclonal antibodies (FITC, PE, PerCp-Cy5.5 and APC) were purchased from BD biosciences or eBiosciences. Different analytical panels were applied on BM, spleen, peripheral blood, skin and intestine after preparation as single cell suspension. The analysis was carried out to detect myeloid and lymphoid population as well as chimerism level.

In almost all of the experiments, 5×10^5 cells were first incubated with 0.5 mg/10^6 cells of MoAb (2.4G2) for 15 min at 4°C and then with the relevant panels of flourochrome-conjugated MoAb (0.5 mg/10^6 cells) for 30 min at 4°C. Finally, cells were washed twice with FBS. For intracellular staining, the stained cells were fixed in 4% formaldehyde washed and incubate for 20 minutes in perm/wash solution at 4°C. Relevant intracellular Abs (FoxP3, or cytokines) were added to the cells, dipped in perm/wash for 30 min according to the manufacturer instructions. Then, cells were washed twice with FBS and the samples were analyzed by FACSCalibur (Becton Dickinson Systems, San Jose, CA, USA) at the same day unless otherwise mentioned in the papers.

3.8 IMMUNOHISTOPATHOLOGY

Tissue samples were fixed in neutral buffered formalin for 24 hours, transferred to 70% ethanol, dehydrated and embedded in paraffin according to standard procedures. Section of 4μm prepared and sections were stained by Hematoxylin & Eosin for histology evaluation.

For Immunohistochemistry evaluation, tissues were embedded in OCT (Histolab, Stockholm, Sweden) and freezed in liquid nitrogen. Detection of CD4 (RM4-5) and CD8 (53-6.7) cells in the tissues were performed using rat anti-mouse monoclonal antibodies. Briefly 4–5 μm sections were cut. Slides were fixed in cold (-20) acetone for 3 minutes. After overnight drying slides were rinsed with PBS and treated with 3% H_2O_2 in methanol and blocked with goat serum in PBS. Primary antibodies applied at 4°C for one hour. After rinsing in PBS, a biotin-labelled secondary goat anti-rat antibody was applied. Sections were incubated with ABC–HRP complex. Binding sites were visualized with diaminobenzidine/hydrogen peroxide, and the slides finally counterstained with hematoxylin.

3.9 DETECTION OF CYTOKINES IN SERUM

Sandwich immunoassays for cytokines were performed using Gyrolab Bioaffy (Gyros AB, Uppsala, Sweden). Briefly, the system consists of a processing station performing all transfers of liquid (samples, reagents, wash solutions, etc.) from a microtiter plate into a CD microlaboratory, Gyrolab Bioaffy, and an integrated detector for laser-induced fluorescence (LIF). The CD microlaboratory contains 104 identical microstructures, connected in groups of 8, to generate 104 data points. The microstructures contain 2 inlets for liquids, individual and common, functions for volume definition (200 nl) and a 15 nl streptavidin column to which selected biotinylated antibodies can be attached followed by sequential addition of samples and antibodies labelled with Alexa Fluor 647 (Molecular Probes, Eugene, OR, USA) to form a sandwich immunoassay. Liquid introduction is facilitated by capillary force and further movement of liquids within the CD microlaboratory is driven by centrifugal force when spinning the CD. Detection of fluorescent signal on column is performed by rotating the CD while moving a LIF detector in the radial direction of the CD. The signal in the column is integrated representing the total response from the reaction. The response is compared with a standard curve processed in an identical fashion and the cytokine concentration calculated using Gyrolab Evaluator software. In total, 15 µl of serum was used for duplicate analysis of IFN-γ, IL-2 and TNF-α. The assay was conducted by sequential addition of biotinylated capture antibodies (200 nl), followed by samples or standards based on recombinant cytokines (200 nl) and finally complementary antibodies labelled with Alexa Fluor 647 (200 nl) were spun over the column. After each addition of reagents or samples to the microstructure, the streptavidin columns were washed repeatedly using 0.01 M phosphate buffered saline, pH 7.2, containing 0.01% Tween-20 (PBS-T). The Gyrolab Bioaffy was also used for determining cytokines in the culture supernatants.

3.10 GENE ARRAY PROCEDURE

3.10.1 **Extraction and purification of RNA**

RNA extraction was performed according to the Affymetrix GeneChip Expression Analysis Manual (Affymetrix Inc., Santa Clara, UK). Briefly, total RNA from tissues were extracted with the Qiagen RNeasy kit (WVR, Stockholm, Sweden) according to the manufacturer's instructions. The integrity of the extracted RNA was confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

3.10.2 **Synthesis of cDNA and microarray analysis**

Double-stranded cDNA was synthesized using 50 ng of total RNA using the Super Script Choice system (Invitrogen Inc). Utilizing cDNA as the template, cRNA was synthesized using an In-Vitro Transcription (IVT) kit (Affymetrix Inc). The cRNA was fragmented in a fragmentation buffer (40 mmol/L Tris-acetate, pH 8.1, 100 mmol/L KOAc, 30 mmol/L MgOAc) for 35 min at 94°C. Fragmented cRNA (15 μg/probe array) was hybridized with the Mouse Genome 430 2.0 GeneChip arrays at 45°C for 18 hours in a hybridization oven with constant rotation (60 rpm). The chips were washed and staining was performed using streptavidin phycoerythrin conjugate (SAPE; Molecular Probes, Eugene, OR, USA), followed by the addition of a biotinylated anti-strepavidin antibody (Vector Laboratories, Burlingame, CA, USA), and finally with streptavidin phycoerythrin conjugate. Probe arrays were scanned using fluorometric scanners (Affymetrix Scanner). The scanned images were inspected and analyzed using established quality control criteria.

3.11 STATISTICAL ANALYSIS

All data are expressed as means±SE (standard error) unless otherwise mentioned. Differences between allogeneic and syngeneic analyzed using Mann-Whitney (U-test). $P<0.05$ is considered statistically significant. Survival curve was plotted using Kaplan-Meier estimates. All statistical analyses were performed utilizing SPSS ver13 software (SPSS Inc.).

4 RESULTS

4.1 BU-CY AND CY-BU CONDITIONING IN SYNGENEIC SETTING

4.1.1 Myeloablative and immunosuppressive effect

Female BALB/c mice were treated with 60 mg/kg of Bu for four days followed by 200 mg/kg of Cy for next two days similar to clinical setting (Bu-Cy). Another group of mice was treated with the same dose of Bu and Cy but in reverse order (Cy-Bu). At day 0 recipient mice were transplanted with 6×10^5 Sca-1+ cells from male BALB/c mice. Bone marrow and spleen cellularity were measured as an index reflecting myeloid and lymphoid lineage at different time points. Bone marrow cellularity at day 0 decreased significantly ($P<0.05$) by 94% and 61% in Bu-Cy and Cy-Bu, respectively, compare to control (Figure 2a). In contrast to the significant differences in myelo-suppressive properties of the two conditioning regimens at day 0, no significant differences in spleen cellularity (immunosuppression) were observed between these conditioning regimens. Spleen cellularity at day 0 decreased by 82% and 80.5% in Bu-Cy versus Cy-Bu group, respectively, (Figure 2b). After SCT spleen repopulation started earlier (day+3) in Cy-Bu compare to Bu-Cy treated mice ($P<0.05$).

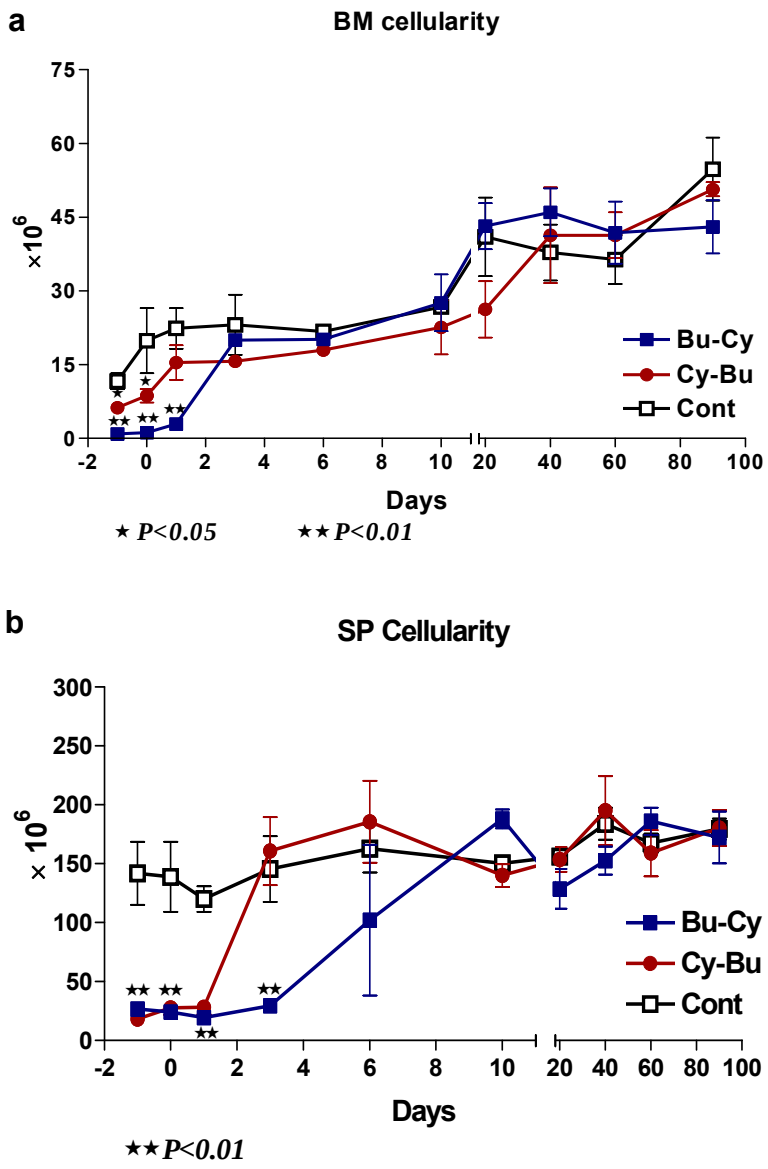


Figure 2. Effect of chemotherapy conditioning (Bu-Cy versus Cy-Bu) on bone marrow and spleen cellularity in syngeneic SCT compared with control mice.

a) BM cellularity **b**) Spleen cellularity

4.1.2 **BM engraftment**

Engraftment and donor chimerism level in the syngeneic setting is considered as an *in vivo* index of myeloablative property of the conditioning regimen. Moreover, durable chimerism (more than 60 days in mice) indicates permanent engraftment of donor stem cells.

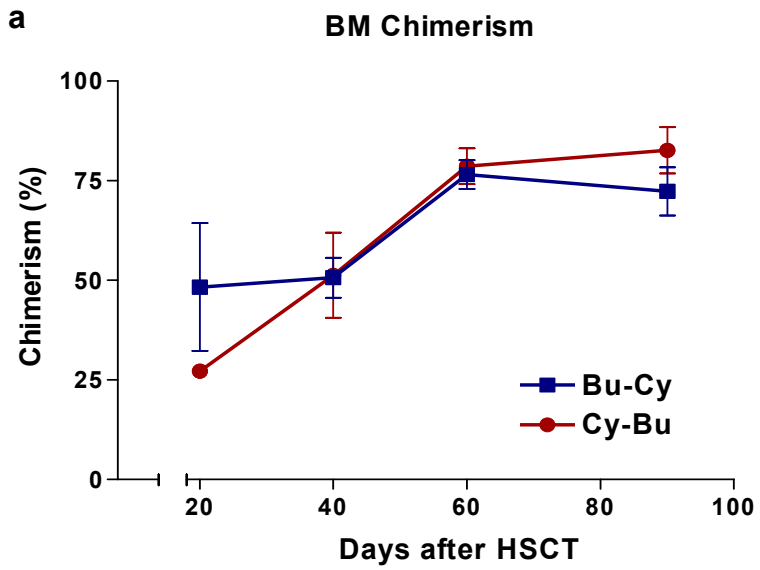
BM chimerism in Bu-Cy and Cy-Bu conditioned mice are presented in Figure 3a. Despite higher chimerism level in Bu-Cy compare to Cy-Bu treated mice at day +20 (48% versus 27%),

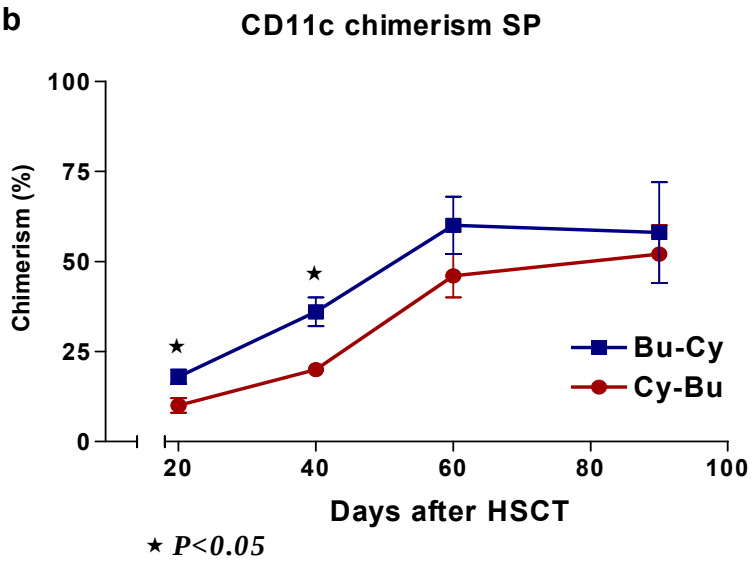
the long term evaluation of chimerism level showed opposite result (72% versus 82% in Bu-Cy and Cy-Bu group, respectively). No significant differences between these two regimens were observed during the studied period. Lower chimerism levels in BM and spleen (data not shown) at day + 20 in Cy-Bu versus Bu-Cy conditioned mice together with early repopulation of splenocytes may indicate that the majority of reconstituted immune cells in the spleen of Cy-Bu treated mice originated from the host.

4.1.3 Dendritic cells (DCs) and T regulatory chimerism

Donor DCs and Treg cells have pivotal role in promotion and regulation of GVHD [153, 192]. We compared engraftment levels of these cell populations between two conditioning protocols. Donor DCs and Treg cells were detected as chromosome Y+ and CD11c+ or CD4+FoxP3+ respectively.

As shown in figures 3b and 3c, the percentage of donor CD11c+ and CD4+FoxP3+ cells in the spleen was lower in Cy-Bu-treated recipients than Bu-Cy treated mice until day 40 after SCT. However, on days +60 and +90, mice conditioned using Cy-Bu exhibited either comparable or even slightly higher chimerism of CD11c+ and CD4+FoxP3+ cells compared to that observed in Bu-Cy treated mice. This data show that donor Treg cells repopulation in Cy-Bu-treated mice occurs later but is higher compared to that observed in Bu-Cy conditioned mice ($P<0.05$).





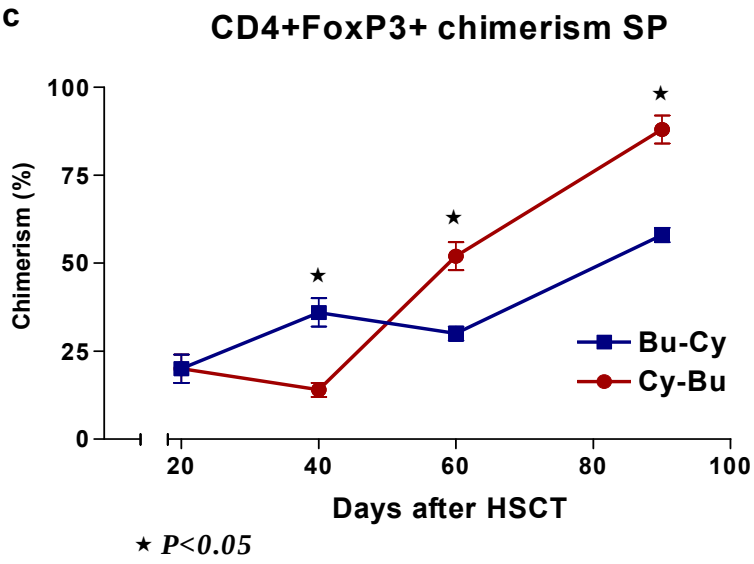


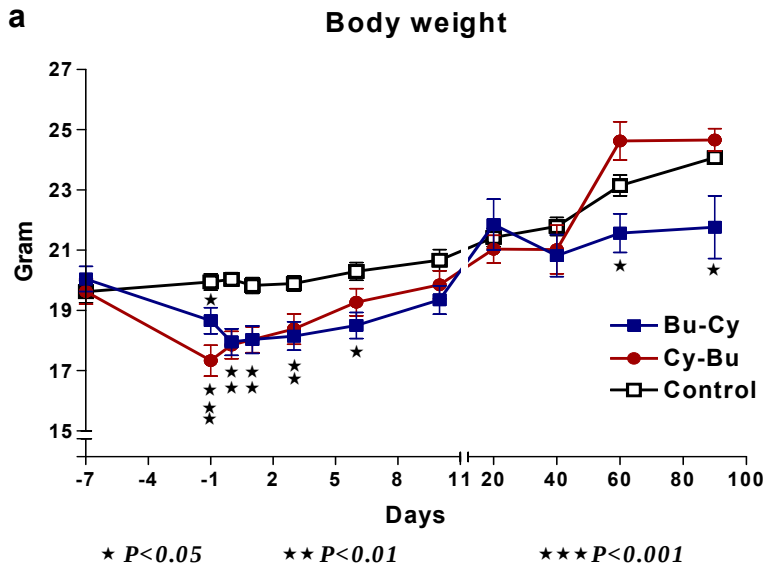
Figure 3. Engraftment and chimerism of DCs and Treg cells. Female BALB/c mice were conditioned with either Bu-Cy or Cy-Bu (60 mg/kg Bu and 200 mg/kg Cy) and transplanted with 6×10^5 Sca-1+ cells from male BALB/c.

a) Engraftment rate of donor stem cell in the BM of Bu-Cy versus Cy-Bu treated recipients in short and long term follow up. **b**) Frequency of donor DCs (chimerism) in the spleen of recipient mice. **c**) Chimerism of CD4+FoxP3+ Treg cells in the spleen of recipients. (■) Bu-Cy (●) Cy-Bu

4.1.4 General and organ toxicity

Weight is a sensitive indicator of general toxicity in mice [239]. We followed the weight changes during conditioning and after SCT in Bu-Cy and Cy-Bu conditioned mice (Figure 4a). Both conditionings decreased weight, however, decrement was higher in Cy-Bu treated mice compared to Bu-Cy up to day -1 ($P<0.05$). The pattern of weight loss during conditioning in both groups indicates that Cy affects body weight more severely than the treatment with Bu since a sharp slope is visible in both groups after the administration of Cy (Figure 4a). Both groups started to recover weight from day+3 after SCT, however, in almost all time points weight was comparable or higher (day+60 and +90, $P<0.05$) in Cy-Bu compared with Bu-Cy treated mice. This might indicate lower toxicity of Cy-Bu conditioning in mice especially in long term follow up.

We examined the effect of reverse order of conditioning on the liver enzymes. As displayed in Figure 4b, long term follow up shows that Bu-Cy conditioned and transplanted mice had higher level of ASAT compared with the Cy-Bu group ($P<0.05$). Additionally the same pattern was observed in the serum level of ALAT, however, difference was not significant (Figure 4b). Increased serum level of liver enzymes, which reflects liver toxicity, together with lower body weight in long term follow up might indicate higher toxicity due to Bu-Cy conditioning compared with Cy-Bu regimen.



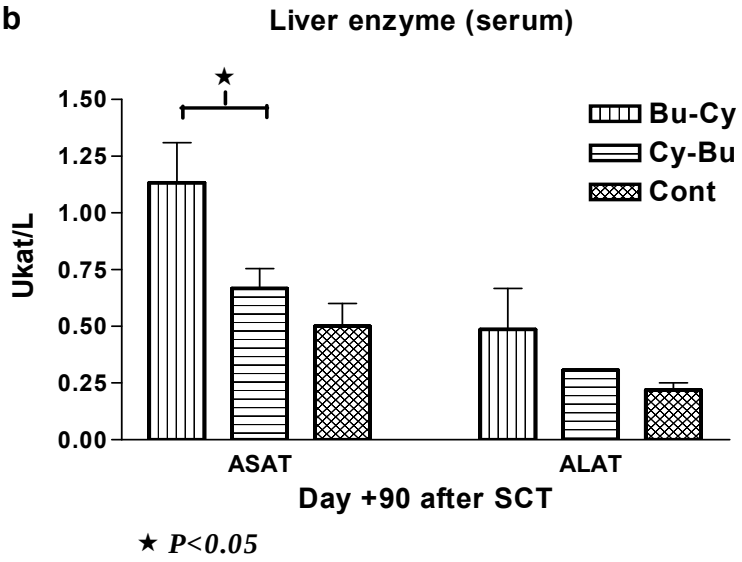


Figure 4. Effect of conditioning regimen on weight and liver enzyme.

Female BALB/c mice were conditioned with either Bu-Cy or Cy-Bu (60 mg/kg Bu and 200 mg/kg Cy) and transplanted with 6×10^5 Sca-1+ cells from male BALB/c. Control group was treated with only vehicle and not transplanted.

a) Weight changes during conditioning and after SCT in Bu-Cy and Cy-Bu conditioned mice compared to control group. (■) Bu-Cy (●) Cy-Bu (□) Control. **b**) Serum levels of ASAT and ALAT, 90 days after syngeneic transplantation in the mice conditioned with Bu-Cy or Cy-Bu compared to control group.

4.2 ESTABLISHMENT OF GVHD MOUSE MODEL

The aim of this investigation was to establish a new mouse model of GVHD using busulfan and cyclophosphamide.

Female BALB/c mice received Bu at doses of 20 or 25 mg/kg/day for four days followed by Cy at the dose of 100 or 150 mg/kg/day for two days. Recipient mice were transplanted using 20×10^6 BM cells plus 30×10^6 splenocytes from either allogeneic (m C57BL/6) or syngeneic (f BALB/c) donor. All study groups are presented in table 2.

Table 2: Treatment groups.

Experimental groups	Conditioning regimens	Recipient	Donor
Group 1	Bu (80mg/kg) +Cy (200mg/kg)	F BALB/c	M C57BL/6
Group 2	Bu (80mg/kg) +Cy (300mg/kg)	F BALB/c	M C57BL/6
Group 3	Bu (100mg/kg) +Cy (200mg/kg)	F BALB/c	M C57BL/6
Group 4	Bu (100mg/kg) +Cy (300mg/kg)	F BALB/c	M C57BL/6
Group 5	Bu (80mg/kg) +Cy (200mg/kg)	F BALB/c	F BALB/c
Group 6	Bu (100mg/kg) +Cy (200mg/kg)	F BALB/c	F BALB/c
Group 7	Bu (80mg/kg) +Cy (200mg/kg)	F C57BL/6	M C57BL/6
Group 8	Bu (80mg/kg) +Cy (200mg/kg)	F BALB/c	---------
Group 9 (Control)	Vehicle (DMSO+NS) Days (-7 to –2)	F BALB/c	---------

Figure 5a shows that >90% of mice in Groups 2 and 4 died shortly after transplantation (4-5 days). All recipients in Group 3 exhibited severe aGVHD and 80% of these mice died within 10 days after BMT.

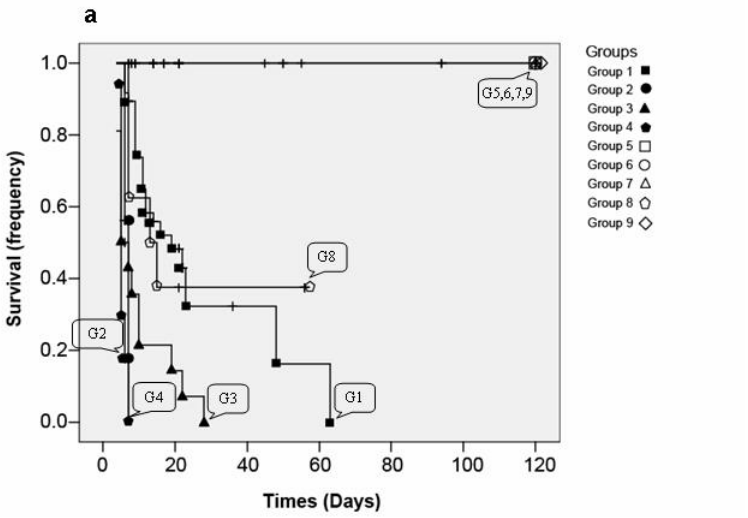
Mice in group 1 conditioned with Bu-Cy (80 and 200 mg/kg) and transplanted with allogeneic BM and SP cells developed aGVHD manifested as hunched posture (Figure 5b), hair loss (Figure 5c,d), ruffled fur (Figure 5d) and most importantly weight loss (Figure 5e) and 100% mortality within 60 days (median survival=11 days) post BMT. This conditioning regimen was considered as optimized protocol of acute GVHD model. Over 60% of non-transplanted mice with this regimen (group 8) died within 21 days, which means that this regimen could be considered as myeloablative. No mortality or signs of GVHD were observed in syngeneic transplanted mice, group 5 and group 6, that received identical conditioning to group 1 and group 3, respectively (Figure 5a).

Based on the results obtained from dose finding study, the conditioning regimen of Bu (80mg/kg) followed by Cy (200mg/kg) was considered as optimal protocol for further investigations.

4.2.1 **Clinical manifestations of GVHD**

Weight loss is an important index of GVHD in mice [239]. Conditioning regimen induced weight loss in recipient mice with nadir at day+3 and +7 in syngeneic and allogeneic setting, respectively (Figure 5e). Syngeneic transplanted mice started to regain weight from day+3 and reached control level within 18 days. Mean body weight at day +5 in allogeneic versus syngeneic group was 16.5±1.2 and 18.4±1.3 g, respectively ($P<0.001$). Allogeneic group continued to loose weight until day+7. The differences in weight between allogeneic and syngeneic settings remained significant until the end of the study ($P<0.001$) (Figure 5e).

We further examined whether this conditioning regimen is strong enough for durable engraftment. Female and male C57BL/6 (CD45.2) mice were conditioned with Bu (80mg/kg) followed by Cy (200mg/kg) and transplanted with 20×10^6 BM cells together with 30×10^6 spleen cells from male C57BL/6 or male B6/SJL (CD45.1), respectively. Chimerisms analyses were evaluated using FISH technique, Y chromosome detection, (Figure 5f) and flow cytometry (CD45.2 versus CD45.1) in the spleen and BM, respectively. Figure 5g indicates durable engraftments of donor cells in the BM and spleen of recipients in long term follow up (day +120).



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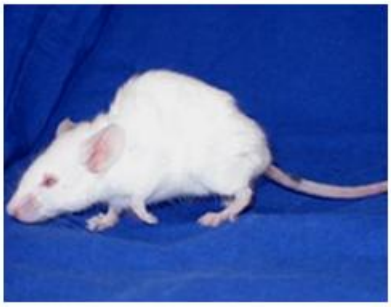

c

d

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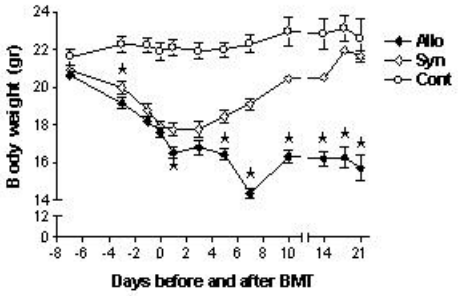
Body weight (gr)
24
22
20
18
16
14
12
0
-8 -6 -4 -2 0 2 4 6 8 10 14 21
Days before and after BMT
Allo
Syn
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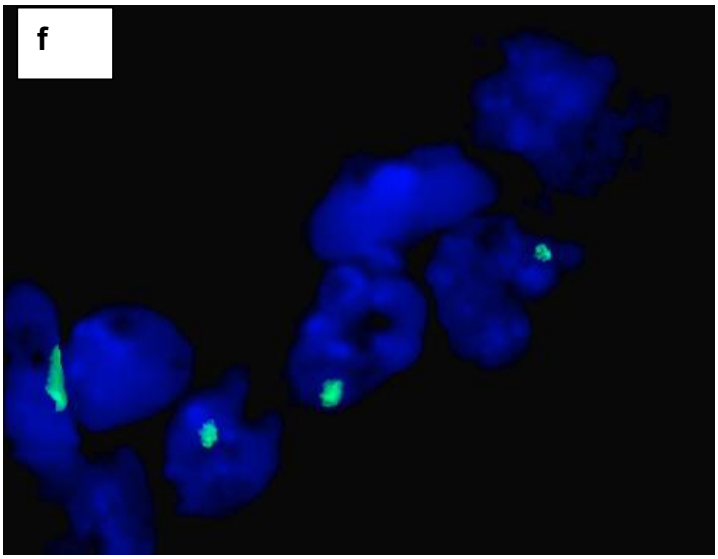
f



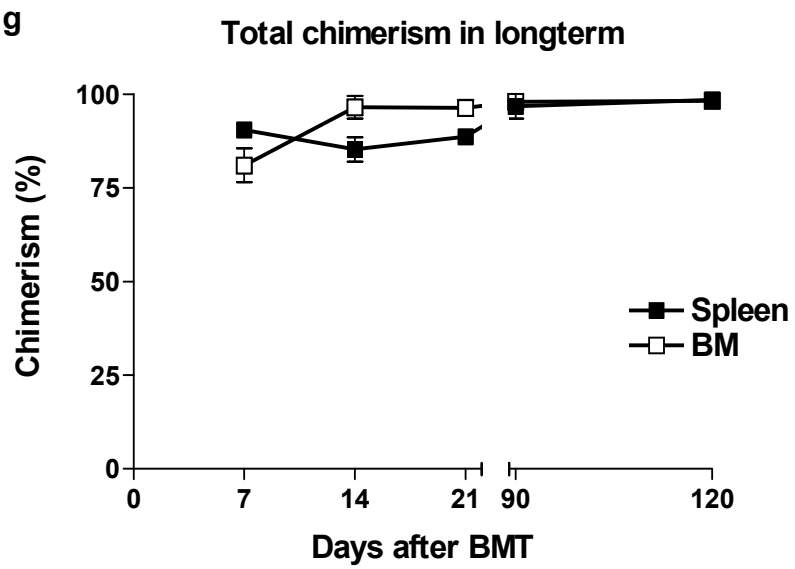


Figure 5. a) Survival analysis of different treatment groups. **Group 1**: Female BALB/c conditioned with Bu–Cy (80–200 mg/kg) and transplanted with male C57BL/6; **group 2**: female BALB/c conditioned with Bu–Cy (80–300 mg/kg) and transplanted with male C57BL/6; **group 3**: female BALB/c conditioned with Bu–Cy (100–200 mg/kg) and transplanted with male C57BL/6; **group 4**: female BALB/c conditioned with Bu–Cy (100–300mg/kg) and transplanted with male C57BL/6; **group 5**: female BALB/c conditioned with Bu–Cy (80–200 mg/kg) and transplanted with female BALB/c; **group 6**: female BALB/c conditioned with Bu–Cy (100–200 mg/kg) and transplanted with female BALB/c; **group 7**: female C57BL/6 conditioned with Bu–Cy (80–200 mg/kg) and transplanted with male C57BL/6; **group 8**: female BALB/c conditionned with Bu–Cy (80–200 mg/kg) and not transplanted; and **group 9**: female BALB/c injected with vehicle (DMSO) only and not transplanted. All transplanted mice received 20×10^6 bone marrow cells and 30×10^6 spleen cells from donor origin.

(b–e) presents manifestation of acute GVHD. Female BALB/c mice were conditioned with Bu–Cy (80 and 200mg/kg, respectively). At day 0 mice received 20×10^6 BM and 30×10^6 spleen cells from male C57BL/6 or female BALB/c (allogeneic and syngeneic setting, respectively). Starting from day +7 the following GVHD manifestations were observed: b) hunch posture, c) hair loss, d) skin lesions, ulceration and denuding were the last manifestation 2–3 days before death and e) weight loss started after conditioning and BMT in allogeneic and syngeneic groups. f) donor cells, Y chromosome-positive in the nucleus, (green spots) in the vicinity of Y chromosome-

negative cells (recipient cells), FISH technique g) long term follow up of engraftment in the spleen and BM of syngeneic transplantation (group7)

All values are Mean±SE. for 3–7 animals in each group per time point. Differences were analyzed statistically using U-test, compared between groups (*P<0.05).

4.2.2 Histopathological features of GVHD

Skin, liver and intestine are considered to be target organs involved in acute GVHD in mice and human. Formalin fixed samples were prepared from different organs at day 0, day +7 and +21. At day 0 bone marrow showed extensive hypo-cellularity and was filled with blood, dilated vessels and some fat cells. Examination of the thymus, primary lymphoid organ that have pivotal role in reconstitution of T lymphocytes, showed that cortex was diminished and devoid while medulla was repopulated with thymocytes. As expected in spleen, extramedullar hematopoiesis was absent in red pulp whereas lymphocytes repopulated the white pulp despite the low cellularity (data not shown).

Figure 6 presents histological changes between allogeneic and syngeneic transplanted mice at day +7 (beginning of tissue damage) and day +21 (established GVHD) in skin, intestine and liver.

Skin. The examination of skin at day +7 and day +21 showed clear differences between syngeneic and allogeneic transplanted mice. At day +7, allogeneic mice displayed fairly extensive skin changes consisting of apoptotic figures, loss of basal living cells layer and disappearance of hypodermal fat layer while syngeneic transplanted mice, displayed an essentially intact epidermis as well as presence of hypodermal fat to a varying extent (Figure 6a). By day +21 syngeneic mice were essentially normalized (Figure 6a), whereas allogeneic mice, showed features of extensive acute GVHD appearing as extensive interfollicular epidermal hyperplasia, combined with hyperkeratosis, extensive, but localized ulcerative changes, intercellular edema in basal layer, frequent apoptotic feature even in the hair follicles and dense infiltrates of neutrophilic granulocytes extending into hypodermal fat (Figure 6a).

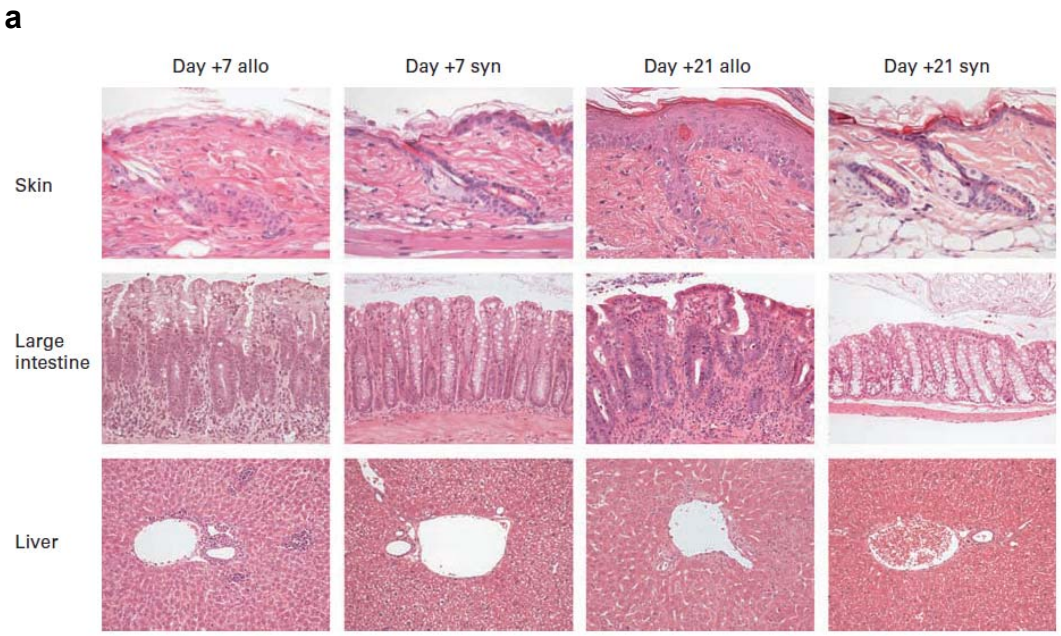
Large intestine. By day+7, allogeneic mice showed clear evidence of colitis demonstrating increased number of inflammatory cells noticeably in the lamina propria and changes in crypt structure (Figure 6a). Further, at day+21 crypts were severely disturbed, showing hyperplasia with numerous cells exhibiting apoptotic features together with frequent mitotic figures. In

contrast, syngeneic mice exhibited near normal structure and few apoptotic cells in both day +7 with more improvement at day +21 (Figure 6a).

Liver. Hepatocytes were generally smaller in the allogeneic group. Although extramedullar hematopoiesis was seen early, by 3 weeks liver structure was normalized in the syngeneic group. The allogeneic transplanted mice primarily showed increased inflammatory infiltrates in the portal triad (Figure 6a).

Immunohistochemistry staining for the presence of T cells as well as occurrence of apoptosis in target tissues confirmed considerable T cells infiltration in skin, intestine and liver during GVHD in the allogeneic setting compared to syngeneic setting (Figure 6b). Furthermore the frequency of apoptotic cells in these tissues was higher in GVHD versus non GVHD transplantation setting (paper II).

Altogether, immuno-histo-pathologic data together with clinical manifestation and dynamism of donor T cells in the spleen of GVHD mice reveal that migration of T cells to target tissues start from day 7 after allo-BMT.



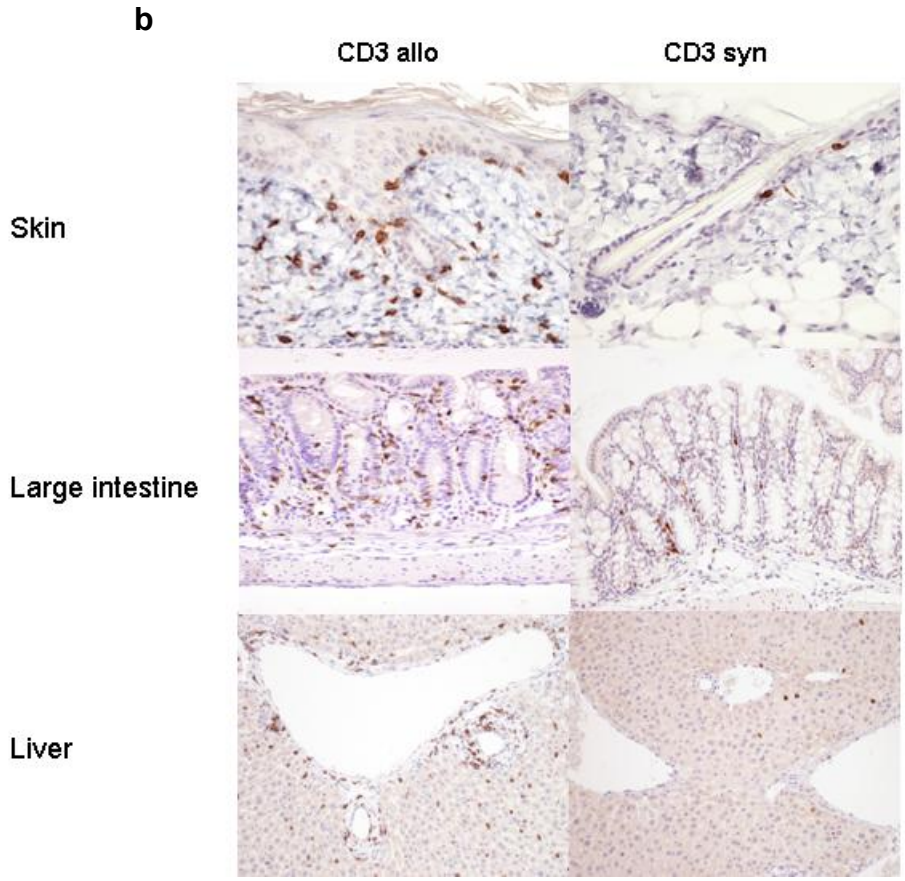


Figure 6. Histopathology sections and immunohistochemistry staining of skin, intestine and liver in allogeneic (GVHD) versus syngeneic transplanted mice.

a) Histology of skin, intestine and liver at day +7 and +21 in allogeneic (GVHD) and syngeneic transplanted mice using chemotherapy as pre-transplant conditioning. **b**) Immunostaining of the skin, intestine and liver at day+21 after BMT in allogeneic (GVHD) versus syngeneic setting. Brown spots indicate CD3+ cells.

4.2.3 **Donor T cells in recipient's intestine and skin**

The presence of donor T cells was examined in the intestine and skin of GVHD mice. Single cell suspension of skin and small intestine was prepared using enzymatic and non enzymatic method, respectively (paper II). Figure 7a presents flowcytometry analysis of single cell suspension from the skin of aGVHD mice at different time points after allogeneic BMT. The frequency of donor T cells (CD3+H-2Kb^{+} cells) among mononuclear cells increased from 4.6% at day+7 to 18.5% at day +21 post BMT (Figure 7a), respectively. Ratio of donor T cells in the skin of recipients was correlated to GVHD severity and skin pathology (Fig 6 and 7a).

The presence of donor T cells was examined in the intestine of GVHD mice. As shown in Figure 7b we confirmed that donor cells exist in the intestine of GVHD mice and more than 90% of them are T cells (Figure 7b).

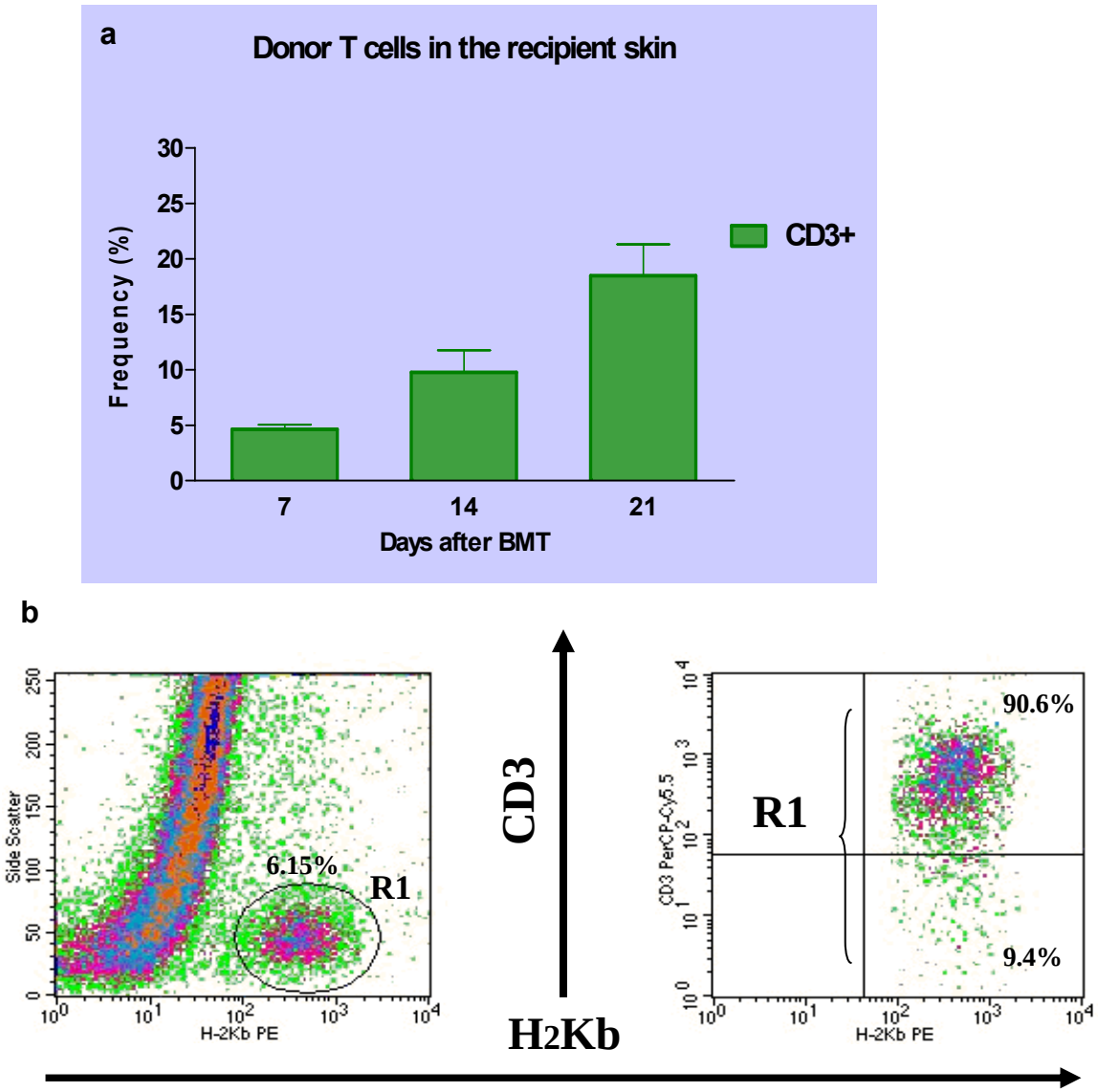


Figure 7. Iinfiltration of skin and intestine by donor T-cells

Female BALB/c mice were conditioned with Bu and Cy at doses of 80 and 200 mg/kg respectively, and were transplanted at day 0 with 20×10^6 BM plus 30×10^6 SP cells from male C57BL/6.

a) After BMT, the infiltration of donor T-lymphocytes (CD3+ H-$2K^b$+) into the recipient skin was detected at days +7, +14 and +21.

b) Twenty one days after BMT, intestinal single cells analysis using FACS showed that donor cells (H-$2K^b$+) are present in the host small intestine. Likewise >90% of infiltrating donor cells were T cells (CD3+).

4.3 EFFECT OF CONDITIONING ON BONE MARROW AND SPLEEN

We evaluated the cytotoxic effect of the conditioning regimen on different cell subpopulations in BM and spleen. The cellularity in BM and spleen decreased by 95% and 88%, respectively, at day 0 (Figure 8 a,b). The decrement of BM and SP cellularity was not distributed equally among different lineage (Table 3).

Considering the results depicted in table 3 two major conclusions could be elicited. First, different lineages that are located in the BM are more sensitive to the conditioning toxicity compared to the homogenous population in the spleen. Second, among different lineages, CD4+ helper lymphocyte, NK, B cells as well as DCs are more sensitive to the conditioning as compared to cytotoxic T cells (CD8+) and lymphocyte with naïve phenotype ($CD44^{low}CD62^{high}$) (Table 3).

Table 3. Effect of conditioning on the immune cell subpopulations of bone marrow and spleen

Time point	*Organ*	*Cellularity*	*T helper*	*T cytotoxic*	*Naïve T cells*	*Effector memory T cells*	*B cell*	*NK cell*	*DCs*	*Myeloid lineage*
Before Conditioning (Day -7)	BM	39±0.5	0.34±0.02	0.11±0.02	0.05±0.01	0.16±0.01	9±0.6	0.6±0.06	0.44±0.04	15±0.32
After Conditioning (Day 0)	BM	2±0.2	0.1±0.01	0.08±0.01	0.04±0.01	0.06±0.01	0.13±0.06	0.05±0.01	0.03±0.004	0.2±0.05
Decrement (%)		95%*	71%*	27%	20%	63%*	99%*	92%*	93%*	99%*
Before Conditioning (Day -7)	SP	189±5.6	39±1.7	14±1.5	23±2.1	14±1.1	69±3.5	10.4±1	5±0.3	-----
After Conditioning (Day 0)	SP	70±2.6	26±0.9	12±0.9	19±1.3	7±0.4	16±2.02	1.4±0.2	1±0.1	-----
Decrement (%)		63%*	33%*	14%	17%	50%*	77%*	87%*	80%*	-----

Female BALB/c mice were treated with busulfan (80 mg/kg) followed by cyclophosphamide (200 mg/kg). Bone marrow and spleen cellularity and immunophenotype of the cells were assessed using flow cytometry before and after conditioning. Cell subpopulations were detected using following markers: helper T cells CD3+CD4+, cytotoxic T cells CD3+CD8+, naïve T cells $CD44^{low}CD62^{high}$, effector memory T cells $CD44^{high}CD4^{low}$, B cells CD19+, NK cells DX5+, dendritic cells CD11c+, myeloid cells CD11b+. Significant difference was assigned with * ($P<0.05$).

4.4 DYNAMICS OF LYMPHOID AND MYELOID LINEAGES

Reconstitution of BM cellularity started immediately in syngeneic transplanted mice and reached to the same level as control mice at day +21. Recovery of BM cellularity in the GVHD mice was significantly suppressed compared to the syngeneic group ($P<0.05$) and did not reach the control levels until the end of study (Figure 8a). The kinetics of the cell recovery in the spleen was slightly different, cellularity in both allogeneic and syngeneic groups increased at day +1 which could be due to settling donor graft. Then spleen repopulation continued in syngeneic recipients, while GVHD mice experienced significantly decreased splenocyte numbers until the end of experiment (Figure 8b).

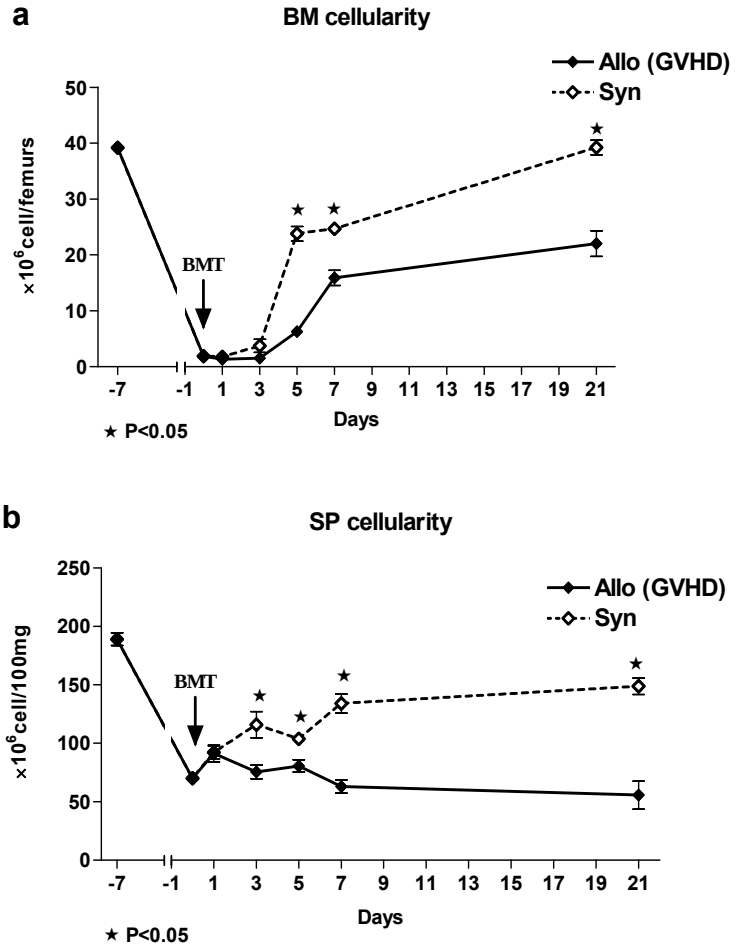


Figure 8. Dynamics of **a**) BM and **b**) spleen cellularity in the syngeneic versus allogeneic (GVHD) transplanted mice after chemotherapy (Bu-Cy) conditioning and BMT.

We investigated the repopulation of lymphoid and myeloid subpopulations in the spleen and BM of GVHD mice comparing with the syngeneic transplanted mice. Figure 9 illustrates the absolute numbers of various cell populations in the spleen (adjusted per 100 mg of SP tissue) and BM. B cells were extremely suppressed in BM of GVHD mice during the whole studied period whereas repopulation of B cells start at day +3 in syngeneic group ($P<0.05$) (Figure 9a). Similar pattern of B cells as in BM was observed in spleen (data not shown). Myeloid cells (CD11b+) that are considered as an indicator for engraftment were also affected at early beginning of GVHD. Reconstitution of myeloid cells in the BM of syngeneic group started at day +1 while reconstitution of myeloid lineage in the BM of GVHD mice started from day +3 (Figure 9b). Except for day +5 we did not find any significant differences in repopulation of CD11b+ cells between syngeneic and GVHD mice (Figure 9b).

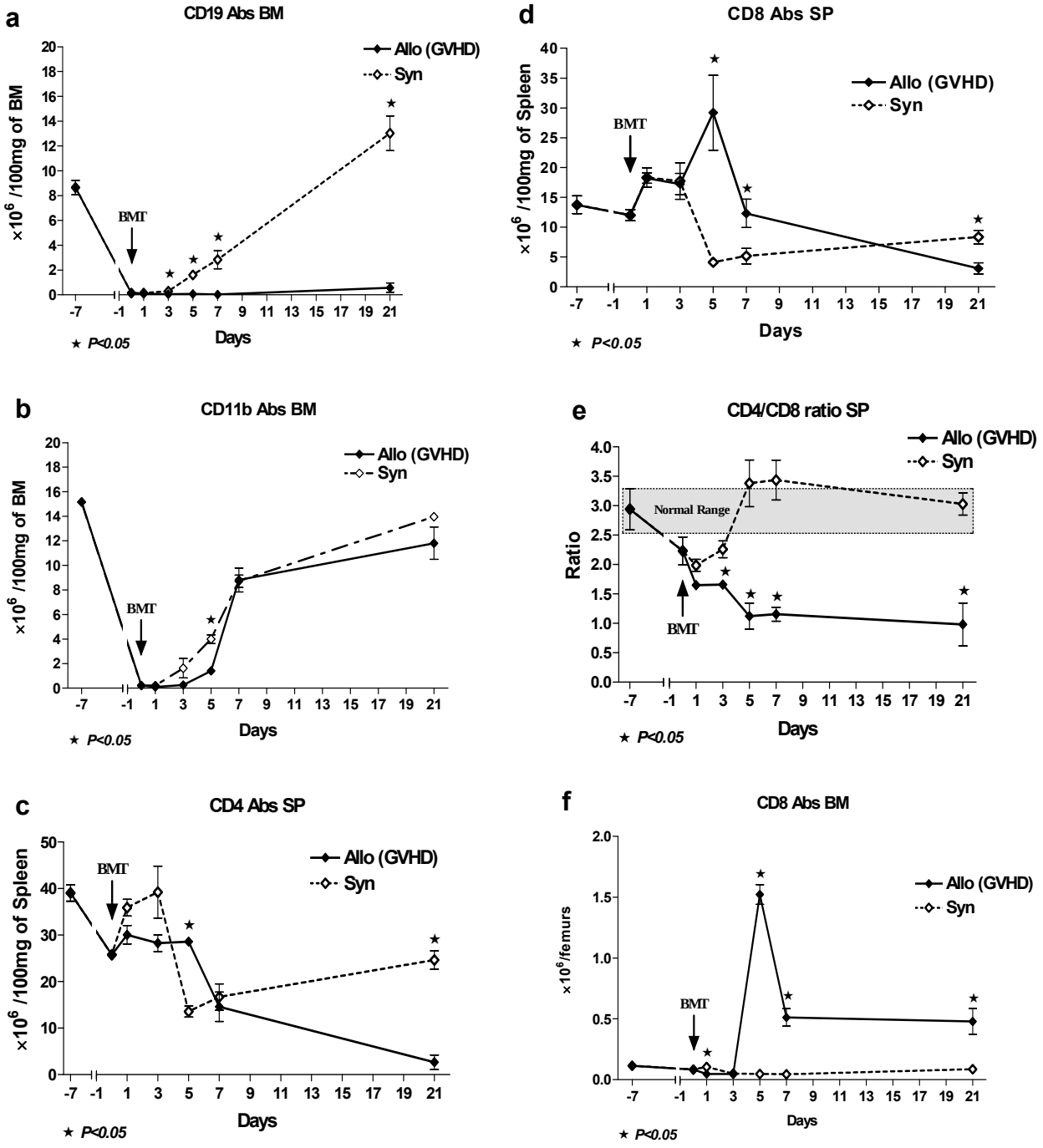
T cells play pivotal role in the pathogenesis of GVHD [46, 47, 213]. We next investigated kinetics of T cell repopulation in the spleen of allogeneic (GVHD) and syngeneic (non-GVHD) transplanted mice. As shown in Figure 9c,d, absolute numbers of CD4+ and CD8+ T cells dropped down after conditioning and then temporary raised in the spleen of both syngeneic and allogeneic transplanted mice until day 3 after BMT (Figure 9c,d). Expansion of CD4+ T cell was higher in syngeneic compare to allogeneic setting. Nevertheless, five days after BMT, the population of both CD4+ and CD8+ T cells significantly decreased in the spleen of syngeneic group as compared to GVHD mice ($P<0.05$) (Figure 9c,d).

Interestingly, at day +5 CD8+ cells in the spleen of GVHD mice expanded vigorously (230% compare to control mice, $P<0.01$) while CD4+ population remained unchanged, between days +1 and +5 and was less than in control (Figure 9c,d). The same pattern od CD8+ cells dynamism was found in the BM of GVHD mice as compared to syngeneic group (Figure 9f) ($P<0.05$). Thus, the CD4+ to CD8+ cells ratio was decreased from early beginning of GVHD until the end of study (Figure 9e) implicating that in our model CD8+ T cells are main initiator of GVHD process.

Starting at day +7, the sizes of both populations (CD4+ and CD8+ T cells) in GVHD mice continuously decreased until they reached to 10% and 30% of controls at day +21, whereas in syngeneic setting recovery of CD4+ and CD8+ T cells reached close to normal level at the same time point (Figure 9c,d).

Dendritic cells (DCs) are known to be the most important cells in triggering of GVHD [62, 153]. We explored repopulation pattern of these cells in both allogeneic (GVHD) and syngeneic

transplanted mice. As shown in Figure 9g, absolute numbers of splenic DCs increased in both GVHD and syngeneic setting one day after BMT. However, three days after BMT, the total number of splenic DCs was significantly increased in the GVHD mice compared to syngeneic and control mice ($P<0.01$) (Figure 9g). Higher number of DCs in the spleen of GVHD mice was persistent up to 5 days after BMT as compared to syngeneic group ($P<0.05$). Seven days after BMT while the number of DCs in the spleen of GVHD mice started to decrease, DCs in syngeneic recipients recovered and reached to normal level by day 21 after BMT (Figure 9g).


a
CD19 Abs BM
Allo (GVHD)
Syn
×10^6 /100mg of BM
BMT
Days
★ P<0.05
d
CD8 Abs SP
Allo (GVHD)
Syn
×10^6 /100mg of Spleen
BMT
Days
★ P<0.05
b
CD11b Abs BM
Allo (GVHD)
Syn
×10^6 /100mg of BM
BMT
Days
★ P<0.05
e
CD4/CD8 ratio SP
Allo (GVHD)
Syn
Normal Range
Ratio
BMT
Days
★ P<0.05
c
CD4 Abs SP
Allo (GVHD)
Syn
×10^6 /100mg of Spleen
BMT
Days
★ P<0.05
f
CD8 Abs BM
Allo (GVHD)
Syn
×10^6/femurs
BMT
Days
★ P<0.05

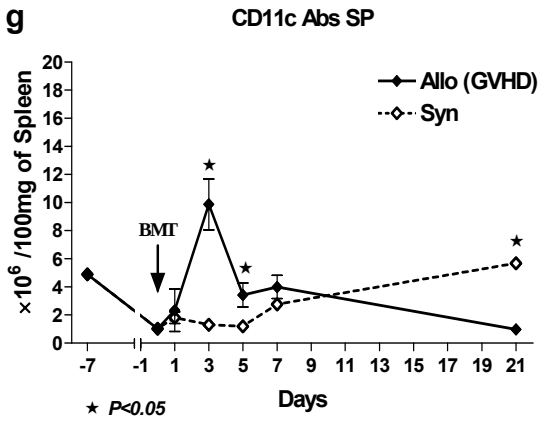


Figure 9. Recovery of different subpopulations in spleen and bone marrow after allogeneic and syngeneic SCT.

Female BALB/c mice were conditioned with Bu (80 mg/kg) followed by Cy (200 mg/kg). Recipients were transplanted at day 0 with 20×10^6 BM plus 30×10^6 SP cells from male C57BL/6 (allogeneic) or female BALB/c (syngeneic) donors. Subpopulations of myeloid and lymphoid cells were assessed in single cell suspensions of spleen and bone marrow using flow cytometry. Cell number per 100 mg of spleen is plotted in the graphs. Results are expressed as mean ± SE.

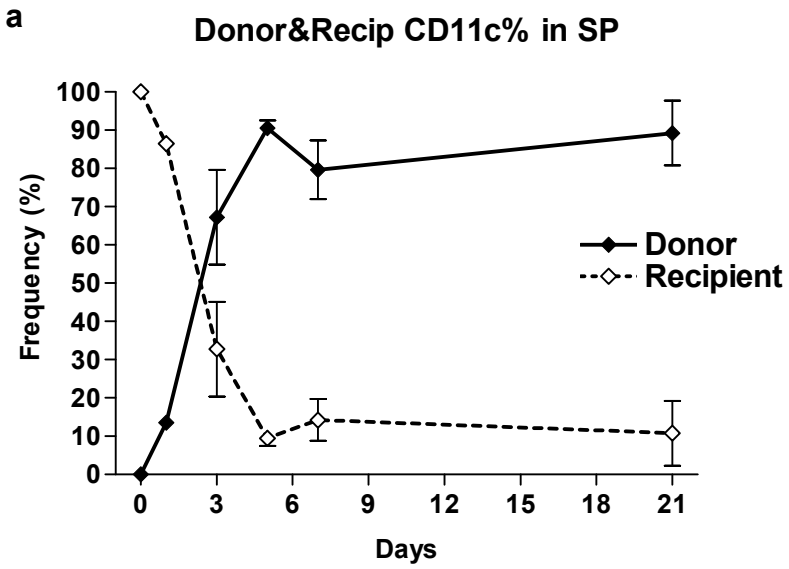
SP = spleen; BM = bone marrow; Allo = allogeneic SCT; Syn = syngeneic SCT.

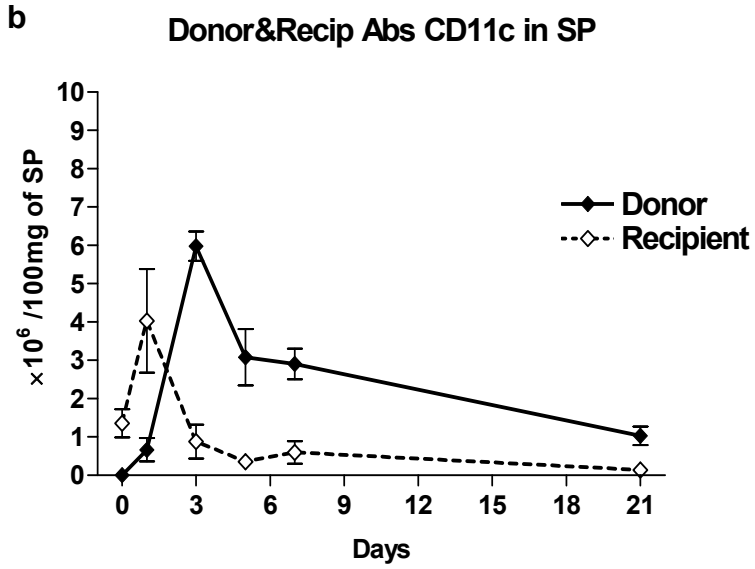
4.5 CHARACTERIZATION OF DCS IN EARLY GVHD

Due to extraordinary expansion of DCs in the spleen of GVHD mice compared to syngeneic transplanted and control mice (Figure 9g), we were interested in exploring the source and activation status of these cells. We analyzed chimerism pattern as well as maturation (activation) status in the splenic DCs of GVHD mice. One day after BMT more than 85% of splenic DCs originated from the recipients (CD11c+H-$2K^d$) while majority (>65%) of DCs at day +3 originated from the donor (CD11c+H-$2K^b$) (Fig 10a). Chronological expansion pattern of DCs in the spleen of GVHD mice is illustrated in Figure 10b. This graph shows that although the early (day +1) expansion of DCs originated from host, the majority of expanded DCs at day +3 belonged to the donor (Figure 10a,b).

Subsequently, we evaluated the maturation (activation) status of identified DCs via measuring the expression level of MHC II (Ia-b) on the surface of these cells. In line with expansion pattern of DCs, Figure 10c shows that expression level of MHC II (one of the

maturation index for DCs) increased by time and reached the maximum level of mean fluorescent intensity (MFI=2389) at day +3 after transplantation in GVHD mice (Figure 10c).





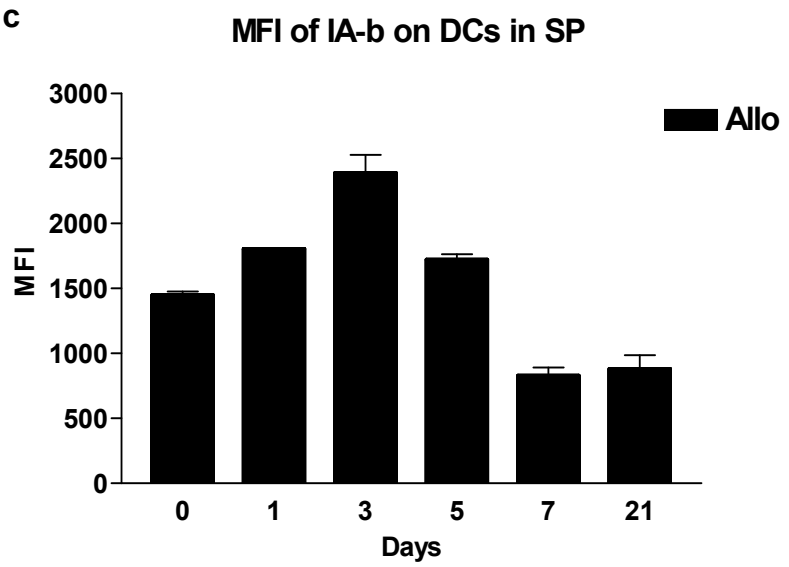
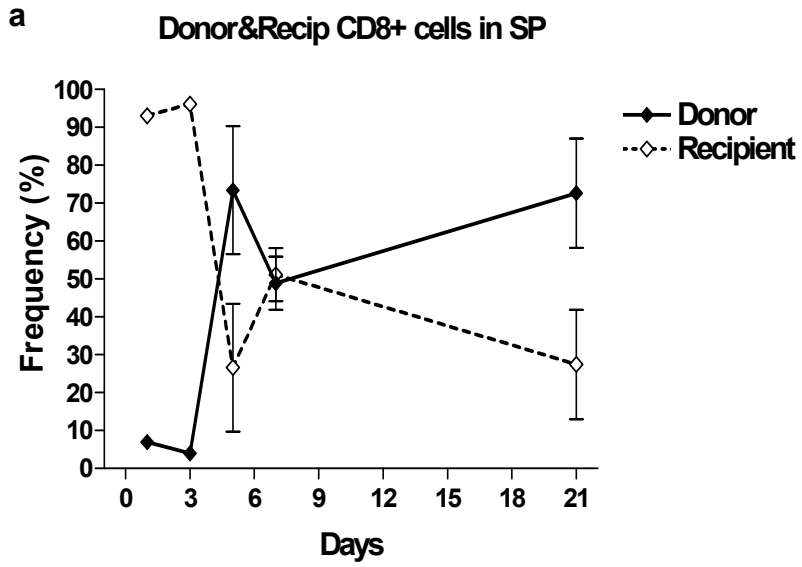


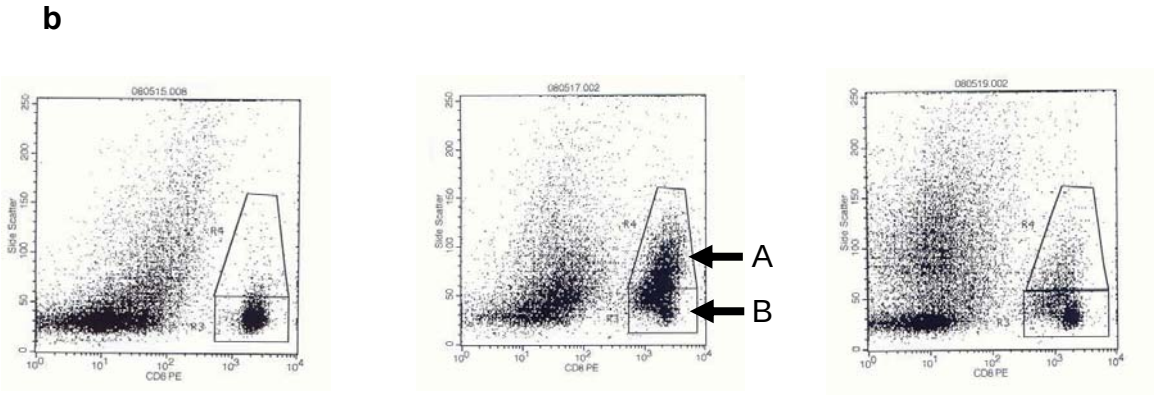
Figure 10. Origin, expansion pattern and activation status of DCs in the spleen of GVHD mice.
Splenocytes of the GVHD mice were analyzed for the **a**) Origin of DCs at different time points after allo BMT. **b**) Chronological expansion pattern of host versus donor DCs in the spleen of GVHD mice. **c**) Expression level of Ia-b (MHC II) on the DCs surface in the spleen of GVHD mice. MFI= Mean fluorescent intensity.

4.6 T CELLS AT EARLY PHASE OF GVHD

Next, we explored the source and activation status of repopulated T cells that emerged 5 days after BMT (two days after DCs expansion) in the spleen of GVHD mice (Figure 9c,d). As shown in Figure 11a, the frequency of donor CD8+ T cells increase from 3.9% at day +3 to 73.5% at day +5, while host T cells decrease from 96% to 26.5% (Figure 11a). These data may be implicated that most of the expanded T cells during this transitional period between day +3 to day +5 originated from donor. In line with this finding, phenotype analysis of splenic T cells in the GVHD mice confirmed emergence of a distinct subpopulation of CD8+ cells at day +5 which was not detectable in syngeneic transplanted mice (Figure 11b, Gate A). This subpopulation is characterized by large granular CD8+ cells in the spleen of GVHD mice 5 days after BMT, however this subpopulation was not detected at day +3 and dramatically decreased by day +7. Interestingly, more than 95% of this large granular lymphocyte (Gate A) originated from the donor (H-2K^b) while small non granular lymphocytes (Gate B) were mixed of donor and host CD8+ cells (Figure 11c). Similar pattern was found in CD4+ cells (data not shown). We then purified these cells (CD8+H-2K^b cells) from both gates A and B. Morphological analysis confirmed that cells from gate A have bigger nucleus as well as more abundant cytoplasm (data

not shown). Disappearance of these subpopulations at day +7 together with the donor origin of these cells is in line with immunohistopathological finding and confirms the fact that infiltration of target tissue is mediated by activated donor T cells at day +7 after BMT.





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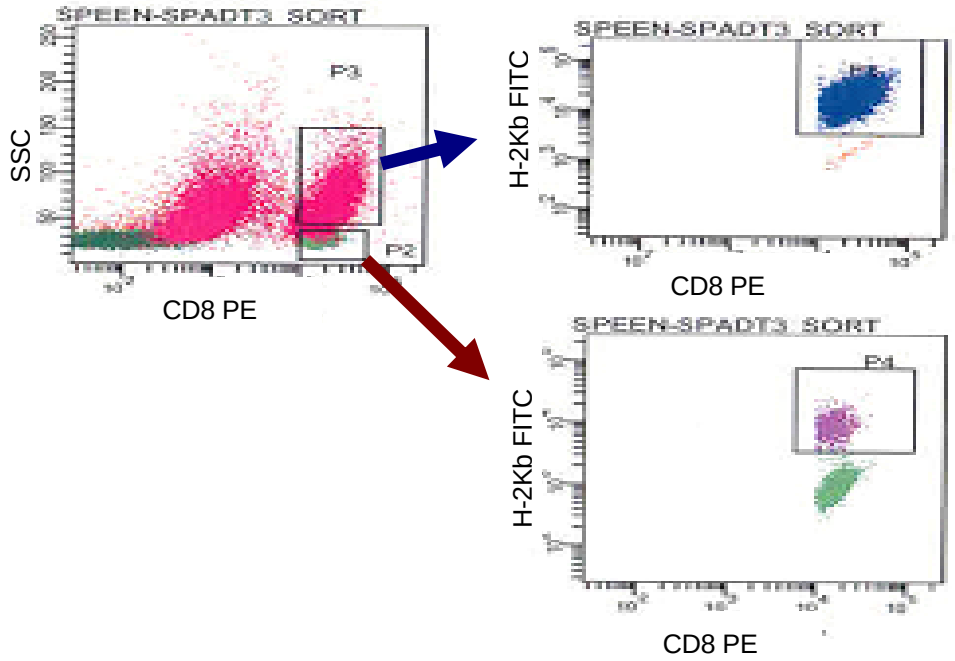


Figure 11. Origin and activation status of T cells in the spleen of GVHD mice.
a) Dynamism of donor versus host CD8+ T cells in the spleen of GVHD mice at different time points. **b**) Emergence of large granulated CD8+ cells (gate A) 5 days after allo-BMT in the spleen of GVHD mice. **c**) Sorting of large granulated (gate A) and small (gate B) indicates that most of cells in the gate A originated from donor (H-2K^{b}) while small CD8+ cells (gate B) are from both donor (H-2K^{b}) and recipient (H-2K^{d})

4.7 DEVELOPMENT OF EFFECTOR/MEMORY

Several reports, with sometimes contradictory data, have shown the role of naïve (CD44^{low}CD62^{high}) and effector/memory (CD44^{high}CD62^{low}) T cells in development and promotion of GVHD [184, 186, 187]. Therefore, we studied the chronological expansion pattern of naïve and effector/memory T cell in GVHD and syngeneic settings. Figure 12a shows dynamics of naïve and effector/memory T cells in the spleen of GVHD compared with control mice. Naïve T cells (CD3+CD44^{low}CD62^{high}) decreased from 68% at day +3 to 31% at day +5, while the frequency of effector memory T cells increased from 17% to 57% at day +3 and +5, respectively (Figure 12a). Increment of effector/memory T cells frequency at day +5 is in line with the peak of T cell expansion as well as emergence of large granular lymphocytes in the spleen of GVHD mice (Figure 9d, 11b,c and 12a). Moreover chimerism analysis of donor T cells implies that most of these effector/memory T cells originate from donor (Fig 11a). In sharp contrast, syngeneic transplanted mice did not show any effector/memory T cell expansion at day

+5 in (Fig. 12b). Moreover, the ratio between naïve and effector/memory T cells were close to control mice (Fig. 12b)

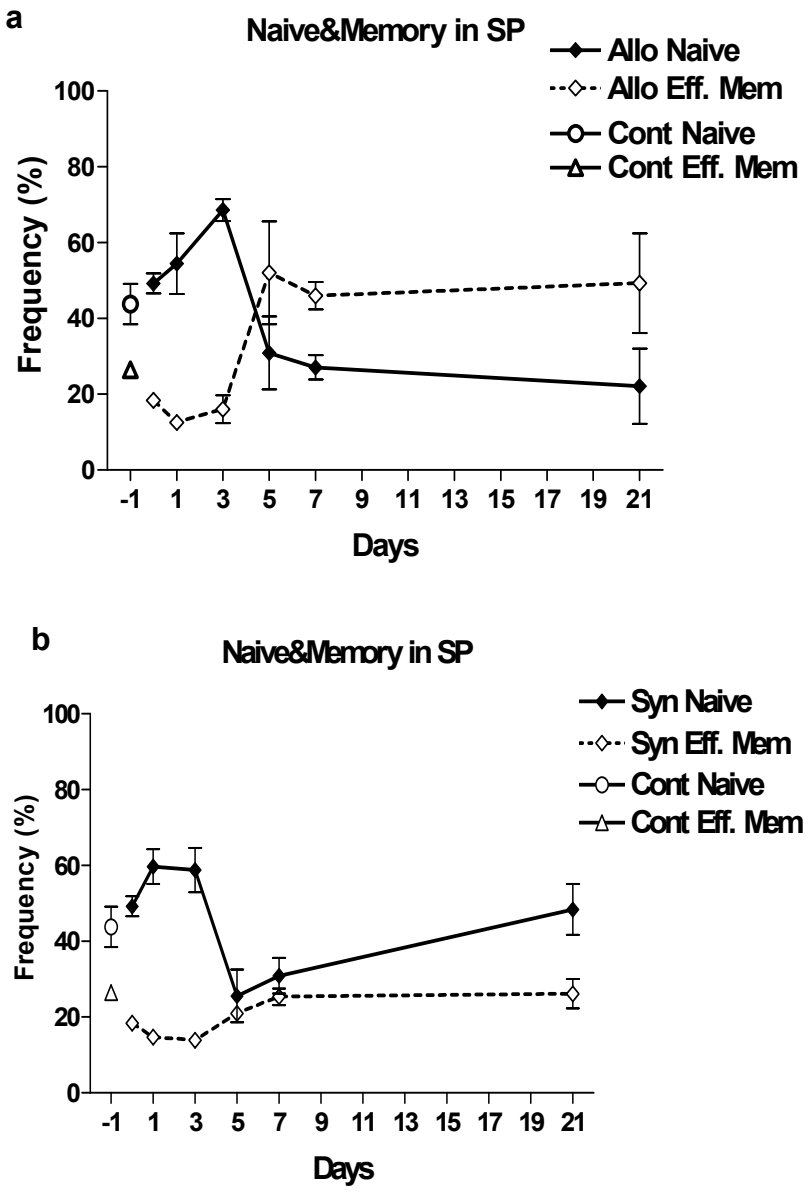


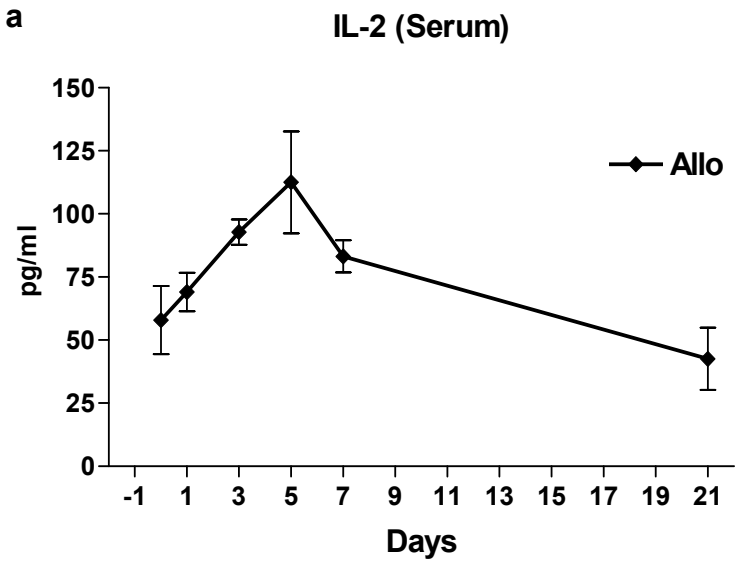
Figure 12. Frequency repopulation dynamism of naïve and effector/memory T cells in the spleen of transplanted mice compared with the control mice.

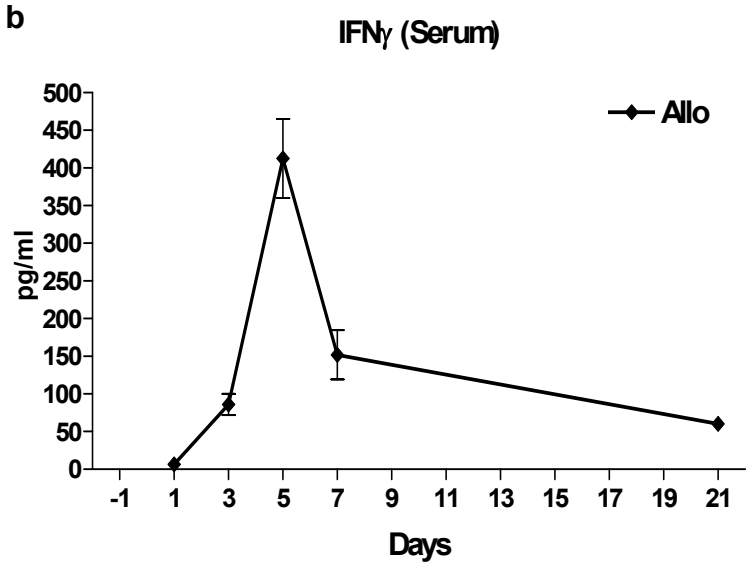
a) Allogeneic (GVHD) transplantation setting; **b**) Syngeneic transplantation settings.

4.8 CYTOKINE PRODUCTION PATTERN AT EARLY GVHD

Inflammatory cytokines have pivotal role in exacerbation of GVHD [87]. Thus, we further determined the kinetic of IL-2, IFN-γ and TNF-α production in the serum of GVHD mice. As

shown in Figure 13a, in agreement with DCs and T cell expansion in the spleen of GVHD mice, serum level of IL-2 increased from 39±13 at day -7 (control mice) to 93±8.7 ($P<0.05$) and 112±20 ($P<0.05$) at days +3 and +5, respectively. Serial measurement of IFN-γ and TNF-α demonstrated similar pattern which means both of these two inflammatory cytokines reached the peak at day +5. This finding is compatible with the appearance of activated granular lymphocyte in the spleen of GVHD mice (Figure 13b,c). None of these cytokines show any increment in syngeneic transplanted mice (data not shown).





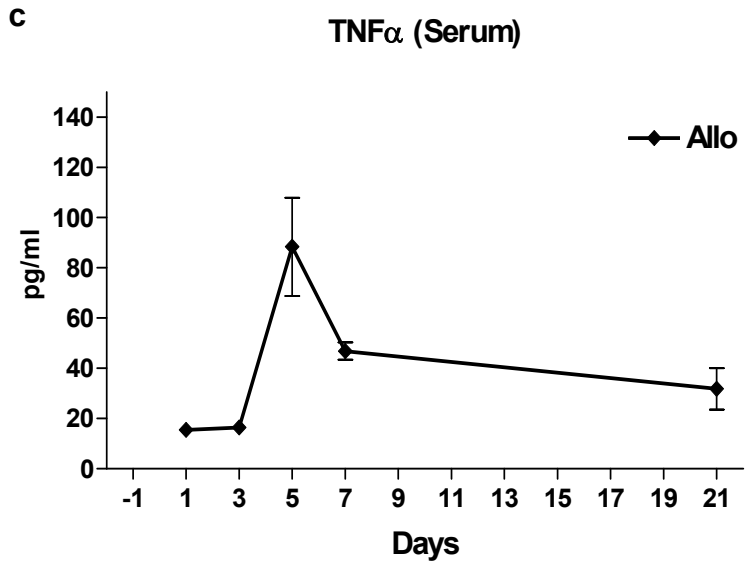


Figure 13. Cytokine levels in serum of GVHD (allogeneic SCT) at different time points. **a**) Interleukin 2 **b**) Interferon gamma **c**) Tumor necrosis alfa

4.9 GENE EXPRESSION PATTERN

4.9.1 **After chemotherapy conditioning**

In the process of stem cell transplantation day 0 considered as a critical time point. To evaluate the toxic effect of conditioning on different tissues, we analyzed the gene expression pattern in the liver and intestine (target organs), muscle (non-target organ) and kidney (possible target organ) at day 0. However the effect of conditioning was more pronounced in the liver as compared to other tissues. The relevant genes in the JAK/STAT pathway were prominently up-regulated in the liver compared to other tissues (Figure 14). The expression of STAT1 and STAT3 was up regulated 2.1 and 2.2 fold in the liver while no increment was detected in other tissues.

Among all expressed genes; antigen presentation pathway, IL4 signaling, B cell receptor signaling and leukocyte extravasations signaling had higher expression in the intestine compare to muscle and kidney after chemotherapy conditioning (Day 0) (Figure 15).

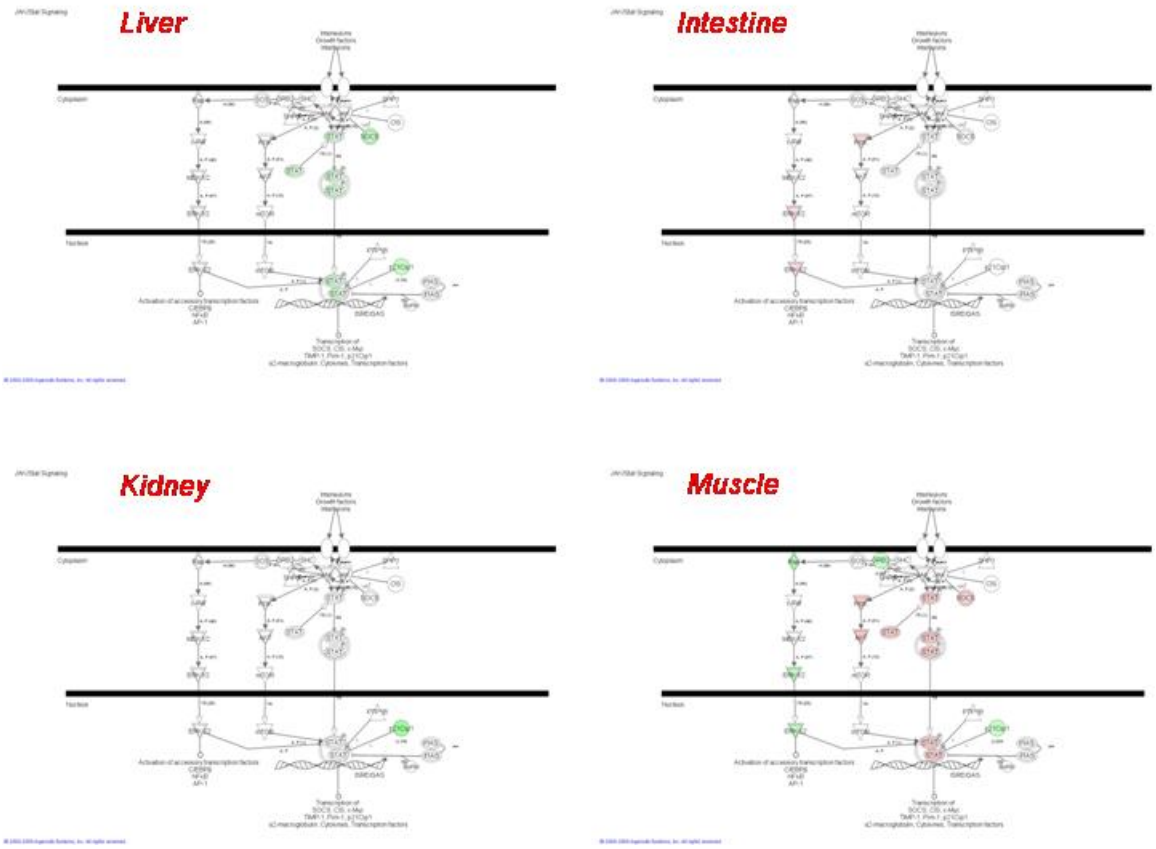


Figure 14. *Comparison of gene expression pattern at day -7 versus day 0*.

Female BALB/c mice were conditioned with Bu (80 mg/kg) followed by Cy (200 mg/kg). Treated mice were killed at day 0 (day of BMT). The circulation system was perfused with PBS containing 2%FBS and 10mMEDTA before sampling of tissues. Liver, intestine, kidney and muscle were snap frozen and gene expression pattern were carried out using Affymetrix Inc. protocol. The analysis showed that JAK/STAT pathway was highly affected and expression of relevant genes were up-regulated in the liver but not in other tissues.

Green color means, genes were down regulated at day -7 (control mice) as compared with day 0 (treated mice).

Red color means, genes were up regulated at day -7 (control mice) as compared with day 0 (treated mice).

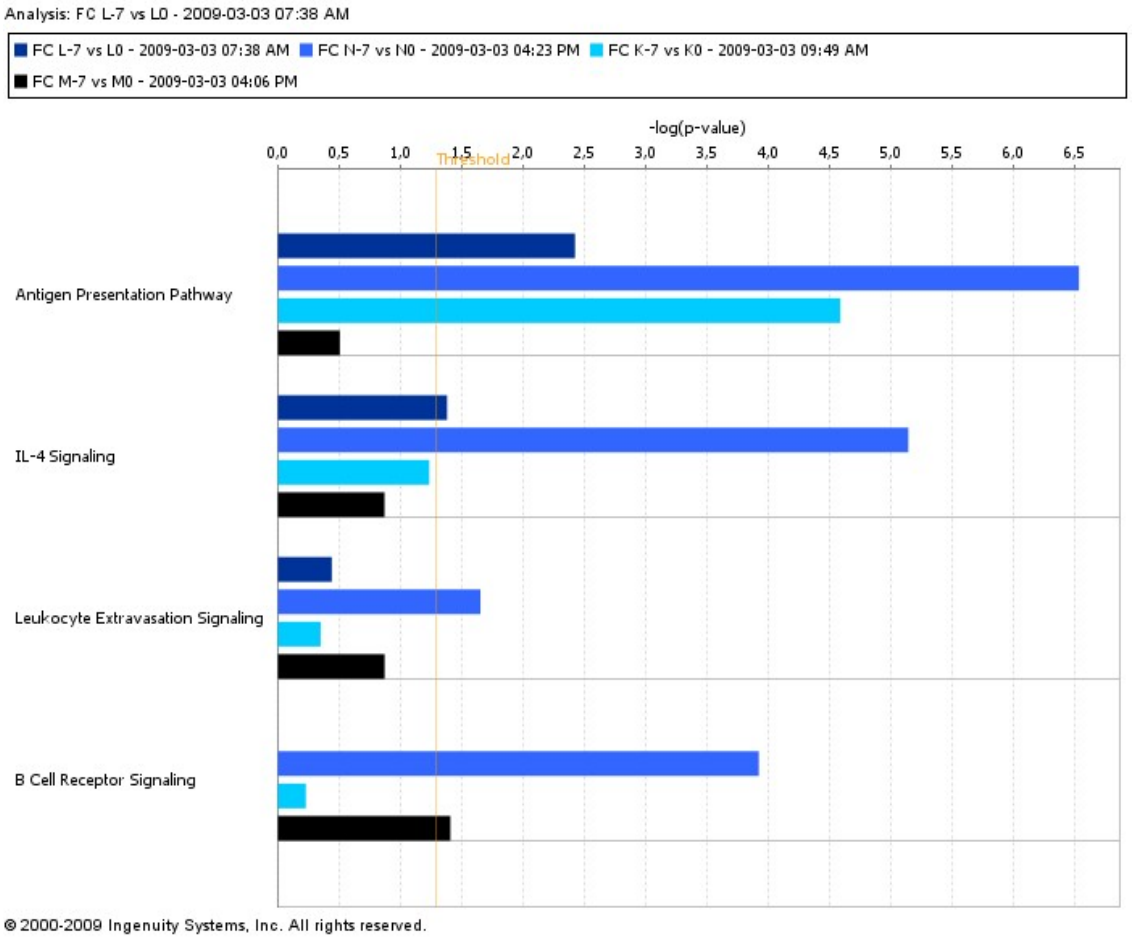


Figure 15. *Effect of conditioning on up regulation of different pathway signaling in different organs of treated mice*. Comparison of Antigen presentation, IL-4 signaling, Leukocyte extravasation and B cell receptor signaling pathways in the Liver (L), Intestine (N), kidney (K) and muscle (M) after conditioning. The differences between the level of relevant gene expression in these pathways were calculated by comparing tissue samples obtained at day 0 with samples from untreated mice at day -7. In these four different pathways intestine is more affected compare to other tissues.

4.9.2 **At the beginning of GVHD (day+7)**

Gene expressions were considerably affected by GVHD compared to syngeneic transplantation setting. Our data shows that 7 days after BMT the gene expression were highly changed in allogeneic tissues compared to syngeneic setting. Especially in antigen presentation, interferon signaling, apoptosis, lymphocyte extravasation and IL-1 signaling pathways. Among the former mentioned pathways; antigen presentation pathway and IFN signaling pathway were highly over expressed in the liver and intestine as well as kidney of GVHD mice compared to

syngeneic setting. The expression of CLIP (CD74) and HLA-DQB2 were 23.2 and 23 fold higher, respectively, in the liver of GVHD mice compared to syngeneic (Table 4). Moreover we have found considerable antigen presentation pathway over expression in the kidney. This finding together with previous data, indicating T cell infiltration in the kidney at late phase of GVHD (data not shown), may imply kidney as a target organ (Table 4).

Symbol	Liver Fold Change	Kidney Fold Change
B2M	----	+5,414
CD74	+23,191	+15,884
HLA-B	+6,335	+32,199
HLA-C	+4,843	+13,692
HLA-DMA	+8,248	+14,682
HLA-DMB	+7,820	+30,869
HLA-DOA	----	+2,298
HLA-DOB	----	+5,081
HLA-DQA1	+7,044	+33,040
HLA-DQB2	+23,022	+25,373
HLA-DRA	+11,786	+23,473
HLA-DRB1	+16,798	+19,250
HLA-E	+2,691	+10,593
HLA-G	+2,255	+4,357
MR1	+2,041	----
PSMB8	+4,829	+44,144
PSMB9	+5,094	+34,056
TAP1	+6,477	+21,255
TAP2	+4,292	+13,829
TAPBP	+3,434	+5,946

Table 4. *Comparison of antigen presentation pathway genes in the liver and kidney of GVHD compared to syngeneic transplanted mice (day+7).* Among them MHC class II showed higher changes in the liver and kidney.

5 DISCUSSION

Graft versus host disease is the major complication of allogeneic HSCT [48]. GVHD is mainly an immunological based disorder and occurs when donor T cells recognize host allo-antigens [55]. However, in the pathogenesis of GVHD, a variety of risk factors can modulate the onset and intensity of manifestations [41]. Improvement of allogeneic HSCT outcome is essentially dependent on the prevention and treatment of GVHD and its complications. Despite progress in understanding of mechanisms underlying GVHD, efficacy of therapeutic approach has not been successful yet [240]. To date, many strategies such as optimizing conditioning regimens [31], inhibiting inflammatory cytokines [94] and/or depleting T cells from the graft [174], have been tried aiming to prevent or reduce GVHD. However, none of these strategies have managed to successfully improve the clinical outcome [55].

An acceptable strategy to reduce the incidence of GVHD for instance, can be to optimize the conditioning regimen, thereby reducing treatment related toxicity, or to use non-myeloablative conditioning [30]. Other possibility is finding new treatment protocols. Busulfan in combination with cyclophosphamide has been used as pre-transplant conditioning for many years [21, 241].

Both Bu and Cy are metabolized through liver enzymes and use GSH for detoxification [15, 32, 34]. Our previous studies have shown that time interval between administration of Bu and Cy influenced the toxicity of Bu-Cy conditioning [35]. Moreover, McCune *et al.* have shown that pharmacokinetics of cyclophosphamide and its metabolites differ between Bu-Cy and Cy-TBI regimens [36, 242]. Considering the above mentioned issues, we hypothesized that switching the administration order of Bu-Cy to Cy-Bu could decrease the regimen related toxicity, whilst maintaining treatment efficacy and engraftment capacity. Using a non-myeloablative regimen of Bu followed by Cy in the syngeneic mouse model of HSCT we have shown that short [243] and long term engraftment (paper I) were comparable between the two regimens (Bu-Cy versus Cy-Bu). However, Cy-Bu conditioning induced a milder, inflammatory cytokine storm at day 0 and also less hepatic and general toxicity [243, 244]. Consistent with our finding Kerbauy *et al.* have reported that Cy-Bu conditioning had less liver toxicity compared to the standard Bu-Cy in the patients undergoing BMT [245]. Moreover, there was no differences in the engraftment level and incidence of GVHD between two groups in their observation [245] (Paper I).

Although, the results were consistent in the syngeneic setting, all of the recipients of allogeneic donor cells conditioned with non-myeloablative Cy-Bu regimen rejected the graft

(data not shown). It could be due to the early recovery of host immune cells in Cy-Bu treated group. However, application of immunosuppressive therapy before and immediately after HSCT might prevent this repopulation of host immune cells. Taken together, futher studies in allogeneic mouse model similar to the clinical setting is necessary (ongoing project).

Aiming to increase the knowledge about the acute GVHD and to find a way to circumvent GVHD development, we have established a new mouse model of BMT and acute GVHD based on myeloablative chemotherapy conditioning (paper II). Although Tutschka and Santos introduced Bu-Cy regimen based on the studies in rat [20], only a limited number of the experimental models of BMT and especially GVHD have been developed using Bu and Cy as conditioning regimen. In spite of the great value and importance of the animal models of GVHD following irradiation-based conditioning, these models can not cover the whole spectrum of clinical settings. Thus, development of new models using chemotherapy as pre-transplant conditioning seems to be necessary.

According to the *ex vivo* and *in vivo* studies, the optimal dose of busulfan required to induce sufficient myeloablation and to permit sustained engraftment was determined to be 100 and 150 mg/kg in mouse [246, 247]. However, in none of these studies the additive effects of Cy together with allo-reactivity of donor cell was investigated. Moreover, estimation of dose of Bu required in mice by converting the doses that are used in clinical setting verified that 80-100 mg/kg of Bu is adequate in the experimental setting. However, the dose of Cy estimated from human dosage in similar way to Bu dose was highly toxic in mice and all of the animals died during conditioning (data not shown). Thus, we have performed a dose titrating study for Bu-Cy conditioning in mice and found that one/third (200-300mg/kg) of the Cy dose in humans produced successful results and induced enough immunosuppression in mice (paper II). Lastly, we have found that administration of 80 mg/kg and 200 (or 300) mg/kg of Bu and Cy, respectively, together with 10 or 20 $\times 10^6$ BM cells and 10 or 30×10^6 splenocyte of C57BL/6 (H-2K^b) induced lethal acute GVHD in the BALB/c (H-2K^d) recipient mice (paper II and unpublished data).

Comparison of manifestations and histo-pathological findings with human and/or other mouse models of acute GVHD from radiation based conditioning, confirmed the accuracy and validity of our model (paper II).

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Besides the conditioning regimen, immune system and allo-antigen disparity have fundamental roles in initiation and promotion of acute GVHD [55, 224]. Donor allo- reactive T cells closely cross talk with donor/ host APCs prime against host allo-antigens and induce tissue damage [55, 224]. Mouse targeted mutations have provided a wealth of information. However, the complexity of genetic backgrounds, the multifaceted mechanisms of GVHD and still not completely known effects of the ablated gene(s) on other immune components, have lead to contradictory findings. Thus, our aim was to analyse the early events of GVHD in an *in vivo*, fully mismatch model of GVHD (Paper II). The reason why we used the fully mismatch model (which is not used in the clinics) was to enhance the intensity of events. Although some reactions would be excessive, less intensive events would become detectable. GVHD is triggered after inflammatory cytokine release (cytokine storm) and stimulation of APCs [87, 88].

Several reports have shown the importance and priority of host/donor APCs in initiation of GVHD [152, 153, 155]. Due to the different fates of donor T cell activation by host or donor DCs it was important to evaluate the role of these cells in an early phase of GVHD development. We have studied the kinetics of immunological events and showed that in allo-BMT, host DCs expand and maturate in response to conditioning and allogeneicity as early as Day+1. However, donor T cell activation will be mainly primed by donor DCs that expand robustly two days later (day +3). Our data (Paper II-III) confirm both observations and in this scenario both host and donor DCs collaborates to provide higher allo-inflammatory environment. Obviously, any of these cells could be able to initiate T cells [62, 153] but the onset and manifestations of GVHD might differ [155].

Several reports stress that during the process of GVHD, donor T cells are activated in the secondary lymphoid organs, migrate to target organs where they induce tissue damage [46, 47, 213]. Meanwhile the role of naïve and/or effector/memory T cells in this process has been studied thoroughly. However, the findings are rather inconsistent [184, 186, 187].

Our analysis (Paper III) of event kinetics demonstrated that at the early phase of GVHD in which T cells do not encounter stimulated DCs (day +1 to +3), the majority of donor T cells have retained a naïve phenotype ($CD44^{low}CD62^{high}$). Upon activation, during later phases of GVHD (day +5 to day +21), and due to interaction with stimulated DCs, naïve T cells are activated and subsequently convert to effector/memory T cells ($CD44^{high}CD62^{low}$). Thereafter effector/memory T cells migrate to peripheral tissues and mediate tissue damage. This observation is supported by Zang *et al.* who have shown that *in vivo* generated effector/memory T cells in the GVHD mice

are able to induce lethal GVHD when transplated to a secondary host (same strain) [186]. It seems that the design of the study and the time of sampling influence the results, warranting further studies.

An important observation in our study was that five days after allo BMT a population of large granulated T lymphocytes (both CD4+ and CD8+) emerged in the spleen of GVHD mice. These cells appeared concurrently with the peak of donor T cell expansion and maximum serum levels of inflammatory cytokines. Of interest, more than 95% of these T cells originated from the donor. At the same time, we observed a part of donor T cells that remained small and non-granulated. Consequently, disappearance of these cells (granulated and large T cells) from the spleen at day +7 implies that these cells most probably are responsible for inducing tissue damage in the process of GVHD. Currently, we are investigating the role of small non-granulated T lymphocytes in the pathogenesis of GVHD together with the probable function of large granulated lymphocyte in the eradication of malignant cells (GVL effect).

Tissue damage in target organs represents the last phase in the pathogenesis of acute GVHD is [224]. The long lasting question is why some particular tissues are exclusively affected by donor allo-reactive T cells. Several reports highlighted the effect of conditioning, inflammation in the up regulation of MHC, chemokines or adhesion molecules in the target organs [106] (Sadeghi *et al.* paper IV). To explore this issue we evaluated the effect of conditioning and early allo-stimulation on the expression of various chemoattractant molecules in the target versus non-target tissues. The gene expression analysis has shown up regulation of inflammatory mediating molecules after chemotherapy conditioning in liver and intestine. This could be due to higher inhabitant immune cells, encountering with bacterial flora or direct damage by cytotoxic drugs (especially concerning the liver where both Bu and Cy are metabolized). Furthermore we have found that the majority of gene expression changes occur after infusing of allogeneic graft. Despite that, better understanding of expression kinetics requires additional time points, especially before T cell infiltration. Moreover, although we couldn't find kidney damage during early phase of acute GVHD, our histopathological and gene expression pattern suggest that the kidney could be a potential target tissue especially in later phases of acute GVHD (paper IV). This finding must be explored in more detail.

6 CONCLUSIONS

The studies involved in the present thesis have shown that:

- The use of chemotherapy as conditioning regimen prior to stem cell transplantation can be further optimized to improve transplantation outcome.
- The newly introduced mouse model of GVHD have brought experimental animal models closer to the clinical setting, and will be a tool for further investigations into the mechanisms underlying GVHD as well as for evaluation of conditioning related toxicity.
- Naïve T cells undergo early allo-activation by host/donor APCs in the secondary lymphoid organs to produce effector/memory T cells that initiate tissue damage in GVHD. Prophylactic therapy should be used prior to donor T cell activation.
- Gene expression analysis was shown to be vastly different in allogeneic compared to syngeneic transplanted mice and was in agreement with the clinical and biochemical changes. Gene expression analysis may be utilized for further understanding of pathophysiology of the disease and hopefully for early diagnosis of GVHD.

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