STUDIES ON THE ENGRAFTMENT AND FUNCTIONAL RECOVERY OF LIVER ACHIEVED BY HEPATOCYTE-LIKE CELLS FORMED BY TRANSDIFFERENTIATION OF ALLOGENEIC BONE MARROW-DERIVED CELLS

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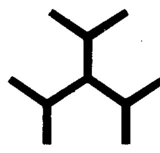

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on the engraftment and functional recovery of liver achieved by hepatocyte-like cells formed by transdifferentiation of allogeneic bone marrow-derived cells"** submitted by Mr. Sumod K in partial fulfillment of PhD degree of Jawaharlal Nehru University, New Delhi, comprises the work done by him at the National Institute of Immunology, New Delhi. This work is original and has not been submitted in part or full for any degree or diploma of any university.

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To

My Parents

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Abbreviations

APC	Antigen presenting cell
aPCC	Activated prothrombin complex concentrates
Asp	Aspartic acid
ATG	Anti-thymocyte globulin
B6	C57BL/6J
B6-GFP	C57BL/6-Tg(UBC-GFP)30Scha/J
bFGF	basic fibroblast growth factor
BM	Bone marrow
BMC	Bone marrow cell
BMSC	Bone marrow stem cell
CD	Cluster of differentiation
cDNA	Complementary DNA
CK-18	cytokeratin-18
CTL	Cytotoxic T lymphocyte
DAB	3,3'-Diaminobenzidine
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DEPC	Diethyl Pyrocarbonate
eGFP	Enhanced Green Fluorescent protein
ESC	Embryonic stem cell
FACS	Fluorescence-Activated Cell Sorting
FCS	Fetal calf serum
FGF	fibroblast growth factor
FIXa	Activated coagulation factor IX
FIX	Coagulation factor IX
Foxp3	Forkhead box 3
FVB-GFP	FVB.Cg-Tg(CAG-EGFP)B5Nagy/J
FVIII	Coagulation Factor VIII
FVIIIa	Activated coagulation factor VIIa
FVIIIa	Activated coagulation factor VIII
FX	Coagulation factor X
FXa	Activated coagulation factor X

GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNF receptor
Glu	Glutamic Acid
GVHD	Graft Versus Host Disease
GVHD	Graft versus host disease
HA	B6;129S4-*F8*^{tm1Kaz}/J [hemophilia A model]
HESC	Human embryonic stem cell
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HVGD	Host versus graft disease
IgG	Immunoglobulin G
IL	Interleukin
IM	intramuscular
IP	intraperitoneal
iPS	Induced pluripotent stem cell
ITI	Immune tolerance induction
IV	Intravenous
kb	kilo base pair
Lin^-	Lineage negative
LR	Liver resection
LSEC	Liver sinusoidal endothelial cell
MACS	Magnetic-Activated Cell Sorting
MHC	Major histocompatibility complex
MiHA	Minor histocompatibility antigen
MQ	Milli-Q
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
NAPQI	*N*-acetyl-*p*-benzoquinone imine
NFW	Nuclease Free Water

NK	Natural killer
NKT	Natural killer T
NOD/SCID	Non-obese diabetic/severe combined immunodeficient
OCT	Optimal Cutting Temperature
OLT	Orthotopic liver transplantation
OSM	Oncostatin-M
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
RO	Retro-orbital
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SC	Stem cell
Ta	Annealing Temperature
TCR	T cell receptor
TEM	Transmission electron microscopy
T_h	Helper T cell
TMB	3,3′,5,5′-tetramethylbenzidine
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cell
vWF	von Willebrand's factor
WT	Wild type
IMDM	Iscove's modified Dulbecco's medium
DMEM	Dulbecco's modified Eagle medium
EDTA	Ethylene diamine tetraacetic acid
BSA	Bovine serum albumin
PE	Phycoerythrin
IgM	Immunoglobulin M
DMSO	Dimethyle sulfoxide
RPMI	Roswell Park memorial institute
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
2-ME	2-mercaptoethanol

MOPS	3-(N-morpholino) propanesulfonic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
cGY	Centigray
HRP	Horseradish peroxidase
TMB	3,3′, 5,5″-tetramethylbenzidine
EGTA	ethylene glycol tetraacetic acid
μm	Micrometers
μM	Micromolar
nM	Nanomolar
mg	Milligrams
cm	Centimeters
kg	Kilograms
ml	Milliliters
μl	Microliters
g	Standard gravity
HAT	Syngeneic transplant recipient
BMT-HA	Bone marrow chimeric mouse
CFSE	Carboxyfluorescein succinimidyl ester
MLR	Mixed lymphocyte reaction
IR	Immunoregulated mice
IS	Immunosuppressed mice
ST	Syngeneic transplantation

Introduction

The major utilities of stem cells at present are in drug development and regenerative medicine. Human stem cells provide an important novel tool for generating *in vitro* pharmacological and toxicological test systems. In the development of new targeted therapies, as well as in critical safety issues, including hepato-, neuro- and cardio-toxicity, animal-based tests are mostly unsatisfactory, whereas the use of *in vitro* model systems is limited by the unavailability of relevant human tissues (Szebényi *et al.*, 2011). Human embryonic stem cell (HESC) lines may be highly valuable in identifying and refining drugs that can have potentially adverse effects on a patient (He *et al.*, 2003; Sharma *et al.*, 2010). HESCs represent an unlimited source for the production of differentiated somatic progenies as they can be maintained at an undifferentiated state *in vitro* (Román-Trufero *et al.*, 2009; Luo *et al.*, 2010) and allow various stable genetic manipulations. As a new opening in personalized medicine test systems, the generation of induced pluripotent stem cell lines and their derivatives can provide patient- and disease-specific cellular assays for drug development and safety assessments (Szebényi *et al.*, 2011).

In an adult individual, organ formation and regeneration were considered to occur through the action of organ- or tissue-restricted stem cells. However, when it emerged that adult stem cells of one organ can give rise to mature cells of another organ, the possibility of using stem cells in regenerative medicine became an attractive option (Forbes *et al.*, 2002). Thereafter, advancements made in stem cell transdifferentiation have led to the continued growth of regenerative medicine. For example, Zhou *et al.* (2008) reported the successful use of *in vivo* induced transdifferentiation of pancreatic exocrine cells in to β cells that could produce insulin and cure diabetes type one. In another study, Ieda *et al.* (2010) transduced fibroblasts with a combination Gata4, Mef2c, and Tbx5 transcription factors. Transplantation of these freshly transduced fibroblasts into the heart of mice showed the formation of functional cardiomyocytes.

Transplantation of hematopoietic or mesenchymal stem cells of bone marrow into an individual, with the intention of assisting regeneration of a diseased organ such as liver or heart, results in directing the cells' differentiation in to cells of that organ. It has been demonstrated that hematopoietic stem cells are able to generate functional hepatocytes after transplantation under conditions favouring hepatic differentiation (Lagasse *et al.*, 2000;

Jang *et al.*, 2004). It has also been demonstrated that transplanted purified hematopoietic stem cells can differentiate in to epithelial cells of skin, gastrointesinal tract and lungs (Krause *et al.*, 2001). It was later found that there exists among the uncommitted bone marrow cells (BMCs), a population of cells that could migrate to the liver in response to an injury and differentiate in to hepatocytes (Khurana and Mukhopadhyay, 2007). Yadav *et al.* (2009) demonstrated the successful utilization of the transdifferentiation potential of BM progenitor cells in curing the X-linked genetic disorder, hemophilia A. Another progenitor BMC population, the mesenchymal stem cells, has been shown to differentiate *in vitro* into cell lines displaying osteogenic, chondrogenic, or adipogenic characteristics (Prockop, 1997). Moreover, they have an immunomodulatory effect on their direct environment (Aggarwal and Pittenger, 2005), and they are able to secrete cytokines that are able to initiate intrinsic tissue regenerative processes (Caplan and Dennis, 2006). Due to their availability and potentially beneficial properties, MSCs have been in the spotlight for regenerative medicine for various indications.

Tumorigenic potential and immunogenecity of the administered progenitor cells are two major concerns with stem cell therapy. As the passage number of a stem cell lines increases, so too does the potential for chromosomal aberrations to arise (Hovatta *et al.*, 2010; Maitra *et al.*, 2005). Embryonic stem cells and induced pluripotent stem cells could form teratomas and, if the cells contain genetic abnormalities, these could potentially develop into teratocarcinomas (Ben-David and Benvenisty, 2011; Blum and Benvenisty, 2008), which are tumors composed of a teratoma element together with persisting undifferentiated stem cells. Another evident safety issue that needs to be tackled by stem cell therapy providers is that of immunogenicity. Although there are reports of immune privilege of human embryonic stem cells (Drukker and Benvenisty, 2004), any foreign cell introduced into a patient will be subject to immune surveillance (Swijnenburg *et al.*, 2008). Zhao and colleagues have recently shown that in mouse iPSCs can induce a T cell-dependent immune response in syngeneic recipients (Zhao *et al.*, 2011).

Stem cell therapy using adult bone marrow (BM) -derived progenitor cells is an attractive option for the control of hemophilia A, which is an X-linked genetic disorder manifested as little or no production of coagulation factor VIII (FVIII) in the affected individual (Yadav *et al.*, 2009). Hemophilic individuals are dependent on external sources of FVIII such as FVIII

concentrates or FVIII substitutes (Lusher *et al.*, 2004; Shirahata *et al.*, 2001). Transplantation of adult BM-derived progenitor cells has the potential of introducing a functional FVIII gene without generating neoplasms or inhibitor antibodies. It also functions to relieve the patient of the necessity for continued administration of injectable pro-coagulants. However, the major problem associated with this technique would be related to the immunological aspect of transplantation. Since the majority of the donors available differ genetically from the recipient, it becomes essential to devise an approach to prevent alloimmune responses of both donor and recipient, which can potentially neutralize the effectiveness of the treatment or, depending upon the severity, even endanger the life of the recipient.

Conventional methods of improving transplantation efficiency and graft acceptance have concentrated on two areas: selection of donor based on genetic homology and prevention of immune complications using immunosuppressive medication. Heightened precautions to counteract the increased vulnerability of the individual to diseases following immunosuppression would mean that the quality of life of a hemophilic does not improve much even after remedial therapy. Thus, it becomes an intriguing question whether it would be possible to utilize the natural mechanisms in immune system to aid in preventing rejection of the transplanted cells. Immune regulation is an integral part of every immune response. Regulatory T cells (T_{reg}s) work to bring an ongoing immune response to an end, to maintain tolerance to self antigens and to prevent autoimmune diseases. By manipulating the recipient T_{reg}s to exert their immunosuppressive effects in the presence of allogeneic donor antigens, it could be possible to prevent rejection of donor cells. Moreover, complementing stem cell therapy for hemophilia A with T_{reg} mediated immune regulation might present a permanent remedy for hemophilia A.

Review of Literature

2.1 Stem Cells

Stem cells (SCs) are undifferentiated cells, able to self-renew and give rise to diverse mature progenies, through the alternation of symmetrical and asymmetrical divisions. SCs exist in all multicellular organisms and play a central role in tissue genesis, regeneration, and homeostasis, by providing new elements to increase tissue mass during pre- and postnatal growth, and by replacing cell loss due to senescence or damage (Piscaglia *et al.*, 2007). SCs possess a hierarchy of potentialities: from the totipotency of the zygote and its immediate progeny, to the pluripotency of embryonic SCs, to the multi/unipotency of tissue-specific adult SCs. The latter persist in terminally differentiated tissues, allowing for their renewal and regeneration (Sell, 2001; Weissman, 2000; Tarnowski and Sieron, 2006). SCs colocalize with supporting cells in a physiologically limited and specialized microenvironment, or niche, which varies in nature and location depending upon the tissue type (Moore and Lemischka, 2006). Niches are dynamic entities, could be redistributed and ideally a candidate niche should be transiently depleted of its full complement of SCs and then shown to take up and maintain a newly introduced SC (Morrison and Spradling, 2008). The reciprocal interactions between SCs and their microenvironment influence SC behaviour through cell-cell and cell-matrix interactions and the secretion of soluble factors (Mimeault *et al.*, 2007).

2.1.1 Transdifferentiation

Numerous papers have challenged the long-held belief that organ-specific SCs are lineage-restricted. In particular, hematopoietic and neural SCs appear to be the most versatile at cutting across lineage boundaries. When a circulating cell engrafts in another organ and assumes some or all of the phenotypic traits of that organ, the process is known as transdifferentiation – the acquisition of a new phenotype. Two decades ago, evidence of transdifferentiation in various settings began accumulating. Mesodermally derived tissues were found to arise from ectodermally derived tissues, and vice versa such as reconstitution of BM from cultured brain cells (Bjornson *et al.*, 1999) and glial cells arising from BM (Eglitis and Mezey, 1997). The pancreatic cell line, AR42J, a subclone of which (AR42J-B13) has been shown, when grown in dexamethasone, to change morphology to a hepatocyte phenotype.

Functional assays for detoxification capacity, *e.g.* cytochrome P450, steroid metabolism and urea synthesis have been demonstrated (Tosh *et al.*, 2002). Schwartz *et al.* (2002) described a population of cells exhibiting a partial hepatocyte phenotype derived from BM. Zhao *et al.* (2003) demonstrated the ability of human peripheral blood monocyte-derived subset to differentiate in to a hepatocyte phenotype.

2.1.2 Hepatic Transdifferentiation of Hematopoietic SCs

Circulating SCs can differentiate into mature hepatocytes *in vivo* (Körbling *et al.*, 2002). In patients with alcoholic hepatitis there is an increase in circulating HSCs when compared with normal controls. De Silvestro *et al.* (2004) demonstrated that peripheral blood HSC levels were elevated after extensive liver resection. It is thought that transdifferentiation happens due to severe organ damage and HSCs can act as a back-up system when an organ's own regenerative capacity is overwhelmed (Forbes *et al.*, 2002). Indeed, therapeutic role of HSCs in liver injury has been described in rodents. Intravenous injection of adult BM cells in the FAH deficient mouse, an animal model of tyrosinemia type I, rescued the mouse and restored the biochemical function of its liver. Moreover, within BM, only rigorously purified HSCs gave rise to donor-derived hematopoietic and hepatic regeneration (Lagasse *et al.*, 2000; Sakaida *et al.*, 2004). Moreover, in support of transdifferentiation, several groups have demonstrated that HSCs can differentiate into hepatocytes without any evidence of cell fusion (Ishikawa *et al.*, 2003; Newsome *et al.*, 2003). The data from our lab showed that a specific subpopulation of BMCs plays an important role in liver regeneration. It was shown that in response to liver injury, these cells get mobilized, home to liver and differentiate into hepatocytes without fusion (Khurana and Mukhopadhyay, 2007). They have also shown the presence of a common progenitor cell for both hepatocytic and hematopoietic lineage in E10.5 foetal liver. These cells which are Ter119^{-}CD45^{+} not only differentiated into albumin and CK-18 expressing cells in the liver of the recipient mice after *in utero* transplantation, but also responded to partial hepatectomy like normal hepatocytes (Khurana and Mukhopadhyay, 2008).

2.2 Liver: Structure and Function

The liver is the largest internal organ providing essential metabolic, exocrine and endocrine functions. These include production of bile, metabolism of dietary compounds, detoxification, regulation of glucose levels through glycogen storage and control of blood homeostasis by secretion of clotting factors and serum proteins such as albumin. These functions of liver that are carried out by the parenchymal cells necessitates the expression and activity of a number of enzymes and a competent cellular machinery within these cells and result in a remarkable heterogeneity of the hepatocytes along the porto-central axis with respect to ultrastructure and enzyme activity (Gebhardt, 1992).

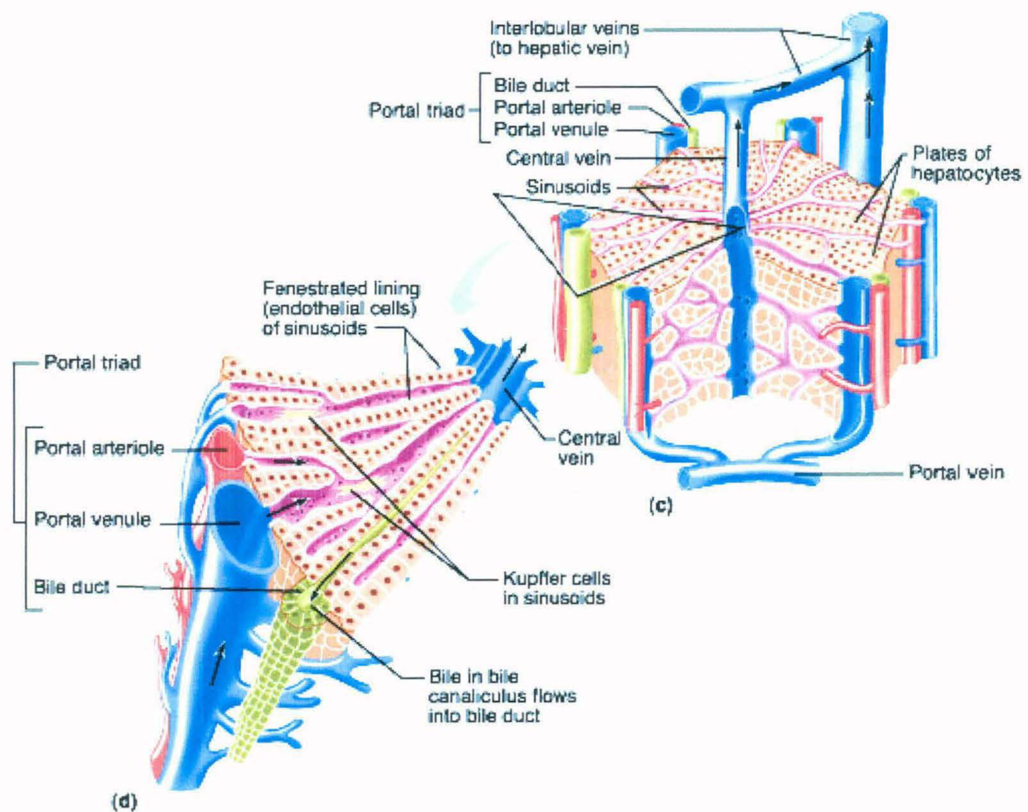

Figure 3.1. Structure of liver lobule: the parenchyma is built up of trabecular network of cell plates made of hepatocytes. The spaces between the plates are called sinusoids and serve as the passage for blood from portal arteriol and portal venule to central vein.

2.2.1 Cells of liver

Liver is interlaced by blood sinusoids, which represent a source of other cell populations essential for liver functions (Figure 3.1). Endothelial cells represent the major cell population found in sinusoids and they mediate communication between hepatocytes and inner space of sinusoids, as well as prevent pathogen infiltration into the liver parenchyma. Moreover, the wall of blood sinusoids is also lined with Kupffer cells, hepatic stellate cells and pit cells (Figure 3.2). All cell populations located within sinusoids also contribute to exchange of metabolites between plasma and hepatocytes, degradation of undesirable particles, such as microbial agents or cellular debris, and regulation of blood flow. The biliary and gall bladder system consists mostly of epithelial cells and it mediates excretion of metabolic waste products into the intestine. Hepatocytes are the principal cell type in the liver accounting for approximately 70% of the mass of the adult organ. Hepatocytes, along with biliary epithelial cells (also known as cholangyocytes) are derived from the embryonic endoderm, while the stromal cells, stellate cells, kuppfer cells, endothelial cells and blood vessels are of mesodermal origin. Based on their localization in lobule, hepatocytes can be divided among periportal and perivenous cells, which differ in their metabolic activity, amount of specific

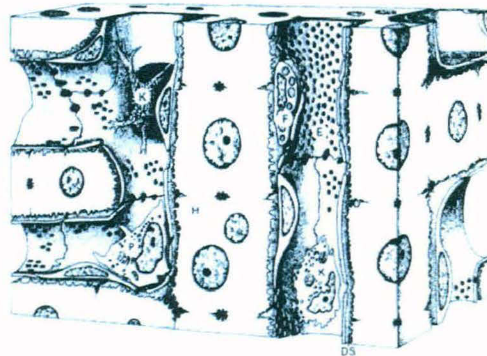

Figure 3.2. Schematic drawing of liver tissue. The fenestrated endothelial cells (E), Kupffer cells (K), stellate cells (F), perisinusoidal space of Disse (DS) and pit cells (P) are depicted. Bile canaliculi are located on the lateral surfaces of adjoining hepatocytes.

intracellular organels and a spectrum of other liver cell populations located in their close vicinity (Kmieć, 2001). Hepatocytes actively participate in the metabolism of proteins,

glycides, lipids, metals and vitamins, as well as in the processes of excretion, detoxification and energy storage. Moreover, hepatocytes may also excrete various mediators acting in paracrine interactions, which may affect functions and communication with distant nonparenchymal cells.

Within the adult liver, the intrahepatic bile duct, portal vein and hepatic artery run in parallel and are referred to as the portal triad. The portal triad is surrounded by hepatocytes arranged in single cell sheets known as hepatic plates, separated by sinusoid spaces that are connected to a network of blood vessels capillaries. Blood plasma from the portal vein enters the sinusoid space and comes into direct contact with the basal surface of hepatocytes.

2.2.2 Liver SCs

The adult liver has a remarkable regenerative capacity and can completely re-grow when up to 70% of its mass is removed (Fausto *et al.*, 2006; Michalopoulos, 2007). However this ability is impaired in numerous diseases such as advanced cirrhosis and hepatitis resulting life threatening liver failure for which organ transplantation is currently the only clinical option. Transplanted hepatocytes also have the ability to repopulate injured recipient livers (Rhim *et al.,* 1994) but the hepatocytes do not significantly repopulate normal adult liver following transplantation. The implantation of isolated hepatocytes or the use of bio-artificial liver devices to provide limited liver function, are also becoming potential options (Dan and Yeoh, 2008; Horslen and Fox, 2004; McKenzie *et al.*, 2008). However, the scarcity of organ donors and the difficulty in culturing adult hepatocytes are serious limitation to this approach.

The adult liver also contains hepatic progenitor cells that are activated when hepatocyte proliferation is inhibited, such as in severe cirrhosis (Fausto, 2004; Oertel and Shafritz, 2008). The hepatic progenitors appear to reside in the small terminal bile ducts and when activated they proliferate giving rise to a cell population called oval cells, which can differentiate into both hepatocytes and biliary epithelial cells (Fougere-Deschatrette *et al.*, 2006; Oertel and Shafritz, 2008). Interestingly, many of the developmental pathways that regulate hepatogenesis in the embryo and involve HGF, FGF, OSM, TNFα and Wnt appear to control

oval cell activation (Apte *et al.*, 2008; Bird *et al.*, 2008; Hu *et al.*, 2007) as well as hepatocyte proliferation during regeneration (Fausto, 2004; Michalopoulos, 2007; Tan *et al.*, 2006b). Although unique liver SC markers are not currently available, oval cells are the best candidates for non-hepatocyte, progenitor-dependent liver regeneration. These cells have a high nuclear/cytoplasmic ratio, termed oval cells due to the initial morphology and their parent cells probably reside in the canal of Hering. Oval cells express markers of both bile duct epithelium (CK-7, CK19, OV-6) and hepatocytes (albumin, alpha-fetoprotein) and have the ability to differentiate into both cell types (Sirica *et al.* 1990; Sirica 1995). Oval cells also express HSC genes (c-kit, CD34, Sca-1 and Thy-1). The cellular sources of liver regeneration often depend on the nature of insult. Although hepatocytes are highly differentiated non-proliferating cells, after partial hepatectomy, or acute chemical injury by carbon tetrachloride or dimethylnitrosamine, they may undergo multiple changes leading to their massive proliferation, thus restoring original liver mass (Sell, 2001). Furthermore, even when the proliferation of hepatocytes is blocked by chemicals like D-galactosamin, alkaloid retrorsine or 2-acetylaminofluorene (Grisham and Coleman, 2002), liver still keeps its regenerative capacity through oval cells.

In humans, the counterpart of oval cells is represented by the intermediate hepatobiliary cells, or ductular hepatocytes, or hepatic progenitor cells, which can be seen in several hepatopathies, such as chronic cholestasis, submassive necrosis, alcoholic liver disease, focal nodular hyperplasia, and liver allograft failure (Zhou *et al.*, 2007). In such conditions, intermediates between hepatic SCs and hepatoblasts and between hepatoblasts and adult parenchyma are observed and amplification of one or both cell subpopulations can occur (Zhang *et al.*, 2008). Like oval cells, hepatic progenitor cells are bipotent, coexpress biliary and hepatocytic markers, and also express hematopoietic progenitor cell antigens (Crosby *et al.*, 2001; Spee *et al.*, 2010).

2.2.3 Xenobiotics and Liver Metabolism

Liver plays a key role in xenobiotic metabolism facilitating excretion of chemicals from body. However, detoxification processes may be also accompanied with increased toxicity of reactive metabolites. Drugs, food additives or numerous environmental pollutants are

xenobiotics daily ingested with food, inhaled or absorbed through skin, thus leading to exposure of various body organs to their toxic metabolites. Xenobiotics enter the cells by either passive or protein-assisted membrane transport and they are metabolised by 2 principal groups of enzymes: i) phase I enzymes, catalyzing reactions of hydroxylation, deamination, dehalogenation, epoxidation or peroxidation, which include monooxygenases, such as of cytochrome P450 enzymes; and ii) phase II (also called conjugation phase) enzymes, which catalyze conjugation of phase I metabolites with the donors like uridine diphosphate glucuronate (glucuronosylation), adenosine-3′- phosphate-5′-phosphosulfate (sulfatation) or glutathione, thus creating water-soluble complexes, which are more efficiently excreted from the body (Nebert and Dalton, 2006).

Although considered safe at therapeutic doses, in overdose, acetaminophen produces a centrilobular hepatic necrosis that can be fatal (Prescott, 1980). Necrosis is recognized as the mode of cell death and apoptosis has been ruled out (Lawson *et al.*, 1999; Gujral *et al.*, 2002). These factors include oxidative stress, nitrotyrosine formation, inflammatory cytokines, and the possible importance of mitochondrial permeability transition. It was shown that acetaminophen is metabolically activated by cytochrome P450 to form a reactive metabolite that covalently binds to protein (Mitchell *et al.*, 1973). The reactive metabolite was found to be *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Dahlin *et al.*, 1984). More recently, the cytochromes 2E1, 1A2, 3A4, and 2A6 (Patten *et al.*, 1993; Chen *et al.*, 1998) have been reported to oxidize acetaminophen to the reactive metabolite. NAPQI is detoxified by glutathione to form an acetaminophen-glutathione conjugate. After a toxic dose of acetaminophen, total hepatic glutathione is depleted by as much as 90%, and as a result, the metabolite covalently binds to cysteine groups of proteins, forming acetaminophen-protein adducts (Mitchell *et al.*, 1973). Adduct formation of critically important proteins could lead to cell death (Nelson, 1990; Tsokos-Kuhn, 1988). Oxidative stress (Dai and Cederbaum, 1995), nitration of tyrosine (Hinson *et al.*, 1998) and upregulation of inflammatory cytokines (James *et al.*, 2003; Bourdi *et al.*, 2002) are other mechanisms that have been found to contribute to acetaminophen toxicity.

2.3 Liver Diseases and Cell Therapy

2.3.1 Inborn errors of metabolism

These are a class of genetic disorders that affect the liver. These are caused by single enzyme defects that result in abnormalities in the synthesis or catabolism of proteins, carbohydrates, or fats. Most are due to a defect in an enzyme or transport protein that alters a metabolic pathway. This group of diseases differs from what is called metabolic disease in the adult or more accurately metabolic syndrome, which includes visceral obesity, elevated triglycerides, elevated fasting blood sugar, and a decrease in high-density lipoprotein cholesterol levels. This group of diseases can be divided into (1) diseases that lead to structural liver damage with liver failure or cirrhosis, with or without injury to other tissues, such as alpha-1-antitrypsin deficiency and cystic fibrosis, and (2) diseases due to a metabolic defect expressed solely or predominantly in the liver but leading to injury to other organ systems. Examples of such diseases include the urea cycle disorders and hyperoxaluria (Hansen and Horslen, 2008). Although individually rare, when considered together, liver-based metabolic diseases are the second most common indication for liver transplant after biliary atresia (Burdelski *et al.*, 1991).

2.3.2 Acquired liver pathologies

Acquired diseases of liver affect hundreds of millions of patients worldwide. The most common causes of hepatopathy are chronic hepatitis C and B, alcoholism, nonalcoholic fatty liver disease, autoimmune, and drug-induced hepatic disorders. Many of these conditions can be prevented or treated, but if not, they can lead to progressive liver injury, liver fibrosis and ultimately cirrhosis, portal hypertension, liver failure, and, in some instances, cancer. Orthotopic liver transplantation (OLT) is the only available treatment for end stage liver pathologies. Since the availability of donor livers are severely limited, a significant number of patients die every year due to lack of treatment (Locke *et al.*, 2009). Given the donor organ shortage, various alternatives to OLT have been evaluated, such as split-liver and related

living-donor liver transplantation. These procedures are still limited by the donor scarcity, the high costs, and immune complications (Fiegel *et al.*, 2006; Di Campli *et al.*, 2003).

2.3.3 Cell therapy in hepatology

Cell therapy has numerous potential advantages when compared to OLT, since transplantable cells can be (1) *in vitro* expanded and cryopreserved, abolishing the limit of organ shortage; (2) genetically manipulated, to correct inborn errors of metabolism; (3) cryopreserved for future use; (4) infused without major surgery, and (5) obtained from the same patient, avoiding risk of rejection and need for lifelong immunosuppression (Piscaglia *et al.*, 2008). However, this is not true in case of metabolic and genetic disorders affecting liver, since introduction of a functional gene is required for correction of the condition.

2.3.3.1 Hepatocyte transplantation

One possible cell therapy source to restore the liver functional mass is represented by adult hepatocytes, which represent a particularly appealing tool, because they are mature and fully functional hepatic cells. Since the first successful hepatocyte transplantation in a rodent model of Crigler-Najjar syndrome, many preclinical studies and clinical applications of this technique have been made to cure metabolic liver disorders and end-stage liver diseases (Kung and Forbes, 2009). In most instances, hepatocyte transplantation has been able to generate a clinical improvement for up to 12 months (Sancho-Bru *et al.*, 2009). In patients with liver failure, hepatocyte-based therapies have also included the use of human or porcine hepatocytes in bioartificial liver devices (Sauer *et al.*, 2003). Despite some encouraging results, the interpretation of these studies is hampered by the limited number and heterogeneity of patients, the lack of controls, the variety in terms of experimental design, outcome parameters, and follow-up duration. Moreover, primary cultured hepatocytes are hard to expand *in vitro* and cryopreserved cells are easily damaged during the freezing-thawing procedure. As a consequence, alternative solutions are being examined in the hepatic cell therapy field. Among these, of particular interest is regenerative medicine, based on the therapeutic potential of SCs (Piscaglia *et al.*, 2008; 2001).

2.3.3.2 Embryonic SCs

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of the blastocyst and can generate any differentiated phenotype of the three primary germ layers (endoderm, mesoderm, and ectoderm), as well as germ cells (Tarnowski and Sieron, 2006). ESCs and their derivatives might constitute an easily available source to obtain a large number of transplantable cells for regenerative treatments. ESCs can be indefinitely maintained in an undifferentiated state, though they seem to develop karyotypic abnormalities over long periods in culture (Piscaglia *et al.*, 2008). It has been demonstrated that ESCs can differentiate *in vitro* towards the hepatic lineage by simple removal of factors that prevent their differentiation, and/or through the exposure to appropriate growth factors. Moreover, in several animal models of hepatic disease, ESC-derived hepatocyte-like cells were able to colonize the injured liver and function as mature hepatocytes (Sancho-Bru *et al.*, 2009; Zaret and Grompe, 2008)

2.3.3.3 Fetal Liver SCs

Fetal liver SCs, also named hepatoblasts, appear when the hepatic endoderm has been specified and the liver bud is growing. Hepatoblasts are bipotent, being able to give rise to both hepatocytes and bile duct cells, and coexpress biliary and hepatocytic markers, such as albumin, alpha-fetoprotein and CK19. Murine hepatoblast cell lines have been established by various research groups and their capacity to repopulate the liver upon transplantation in animal models has been extensively proved (Ogawa and Miyagawa, 2009). In contrast to adult liver, ESCs and fetal liver SCs are thought to be highly proliferative, less immunogenic and more resistant to cryopreservation. However, ethical issues and the possibility of immune rejection and teratoma/ teratocarcinoma formation in the recipients are major barriers to their use in current clinical practice (Wu *et al.*, 2007).

2.3.3.4 Induced Pluripotent SCs

The recently described induced pluripotent SCs (iPSs) might circumvent the ethical concerns and the risk of rejection related to embryonic and fetal liver SCs. Indeed, iPSs are embryonic

like SCs derived from somatic cells by forced expression of reprogramming factors such as Oct3/4, Sox2, Klf4,c-Myc, Nanog and Lin28. Theoretically, iPSs could be obtained from the same patient and used for tissue replacement or gene therapy. The first step of hepatic development from iPSs is the induction of definitive endoderm by using activin A. Further treatment with BMP-4 and bFGF can then direct cells towards the hepatic lineage (Zaret and Grompe, 2008). iPS-based cell therapies have been applied in several animal models of pathologies, with encouraging results, and human iPS cells have been demonstrated to possess a hepatocyte-lineage differentiation potential comparable to that of ESCs (Si-Tayeb *et al.*, 2010). Even if some limitations still remain (i.e., the potential for teratoma formation), iPS-derived hepatocytes are a very promising population for cell therapies in hepatology.

2.3.3.5 Placental/umbilical Cord Blood SCs

Another promising source for SC based treatments in hepatology may be represented by cells established from placental/cordonal tissues, which do not seem to form teratomas or teratocarcinomas in humans, and have higher proliferation and differentiation potential than adult SCs. Several studies indicated that umbilical cord and umbilical cord blood, placenta and amniotic fluid are an easily accessible source of pluripotent SCs, which may be readily available for transplantation, or for further expansion and manipulation prior to cell therapies (Mimeault *et al.*, 2007; Gilchrist and Plevris, 2010). These cells can be extensively expanded without loss of potency and have a broad differentiation potential, since they can generate progenies of all three germ layers. These pluripotent annex SCs can be forced to differentiate into hepatocyte-like cells *in vitro* and are capable of liver repopulation *in vivo*, upon transplantation in animal models (Sancho-Bru *et al.*, 2009; Lorenzini *et al.*, 2008). It has been demonstrated that human umbilical cord blood SCs were able to colonize the liver and differentiate into hepatocytes after acute toxic liver damage in NOD/SCID mice and in immunocompetent rats (Piscaglia *et al.*, 2005; Di Campli *et al.*, 2004).

2.3.3.6 Extrahepatic Adult Stem Cells

Liver regeneration is mainly an endogenous process, driven by hepatocytes and resident hepatic stem/progenitor cells. However, it has been observed that certain populations of

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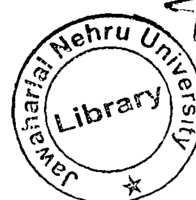


extrahepatic adult SCs can migrate into the liver and contribute to its repopulation and turnover (Piscaglia *et al.*, 2008). Particularly, a high degree of plasticity has been shown by bone marrow stem cells (BMSCs), which can give rise to a wide range of phenotypes, including hepatocyte-like cells. Since the pioneering study by Petersen *et al.* (1999), numerous reports and reviews have been published on BM contribution to liver regeneration, often with contradictory conclusions (Gilchrist and Plevris, 2010; Thorgeirsson and Grisham, 2006; Eckersley-Maslin *et al.*, 2009). It is generally agreed that BM represents a possible source of liver stem cells, even if the frequency of colonization, in the absence of a strong selective pressure, is very low, unlikely sufficient per se to achieve a significant contribution to hepatic repopulation. However, the few BM-derived cells which do engraft may play an important role in modulating the endogenous repair mechanisms within the hepatic SC niche (Piscaglia *et al.*, 2008; Kallis *et al.*, 2007). It has been observed that BMSCs may or may not play a critical role in liver regeneration, depending upon the experimental setting (Oh *et al.*, 2007). Regarding the mechanisms underlying BMSC plasticity, upon engraftment BMSCs might either transdifferentiate into parenchymal cells or fuse with resident cells in the host tissue. Fusion phenomena between BMSCs and other cell types (i.e., Purkinjie cells, cardiomyocytes and hepatocytes) have been shown both *in vitro* and *in vivo* (Piscaglia *et al.*, 2008) Cell fusion is a physiological phenomenon in certain districts, such as liver and muscle, and it may or may not play a prominent role in SC plasticity, depending on the model of injury and the host phenotype (Quesenberry *et al.*, 2005). It has been also proved that fusion and transdifferentiation can coexist and produce therapeutically beneficial results (Tanabe *et al.*, 2004; Masson *et al.*, 2004). Transdifferentiation of lineage uncommitted BM cells in to hepatocytes in paracetamol induced liver injury model has been demonstrated by Yadav *et al.* (2009). In this study it has been clearly demonstrated that cell fusion is not a prerequisite for generation of donor derived hepatocytes.

In order to initiate a BM response, the injured liver must signal to the responding cell types. A pivotal role in BMSC recruitment is played by SDF-1. BM-derived liver-committed SCs expressing SDF-1 receptor (CXCR4) are present in the peripheral blood and may colonize the damaged liver by following a SDF-1 gradient (Ratajczak *et al.*, 2004; Hatch *et al.*, 2002). Other molecules secreted by the injured hepatic milieu that can contribute to BMSC recruitment and homing into the liver are the hepatocyte growth factor (HGF), some fibrosis mediators,

such as matrix metalloproteinase-9 (MMP9), and the G-CSF (Kollet *et al.*, 2003; Lei *et al.*, 2010). IL-8, a known neutrophil chemo-attractant in liver disease, also has the potential to induce the release of HSCs into the peripheral circulation via an indirect mechanism requiring the activation of circulating neutrophils and the release of MMP-9 (Pruijt *et al.*, 2002). The mechanisms underlying the adhesion and retention of BMSCs to human liver compartments have been only in part elucidated (Crosby *et al.*, 2009). Adult BM comprises two main populations of SCs able to convert into hepatic cells, either by fusion or transdifferentiation: HSCs and mesenchymal SCs (MSCs). HSCs are responsible for the renewal of blood cells and can be also isolated from umbilical cord blood and peripheral blood. It is generally accepted that the most primitive and long-term human HSCs are characterized by the expression of CD133, Thy1 (CD90) and VEGFR2 and by a variable expression of CD34 and CD38 (Bryder *et al.*, 2006). BM-resident HSCs can be mobilized into the peripheral blood under specific stimuli such as tissue injury or administration of G-CSF. Mobilized HSCs can colonize extramedullar sites and participate to their regeneration, by promoting the immune response and/or by transdifferentiating into adult SCs within peripheral tissues (Piscaglia *et al.*, 2008; Ratajczak *et al.*, 2004). MSCs, also called stromal SCs, mesenchymal progenitors, or mesenchymal stromal cells, are highly proliferating, adherent cells, that reside in a perivascular BM niche and also in the wall of blood vessels within most organs (Tocci and Forte, 2003). Numerous studies have demonstrated that MSCs are able to differentiate into a variety of mesodermal cell lineages (osteoblasts, chondroblasts, adipocytes, myocytes, and cardiomyocytes), as well as nonmesodermal cells (such as hepatocytes and neurons), depending upon the microenvironment in which they reside (Jiang *et al.*, 2002). MSCs might become a more suitable source for SC-based therapies than HSCs, because of their immunological properties: MSCs are less immunogenic and can induce tolerance upon transplantation (Lysy *et al.*, 2008). Moreover, MSCs showed the highest potential for liver regeneration compared with other BM cell subpopulations in an animal model of hepatic injury (Cho *et al.*, 2009). A more recently identified SC population within the BM, the multipotent adult progenitor cells, seems to be endowed with an impressive plasticity and has shown liver differentiation potential both in culture and in animal models (Kallis *et al.*, 2007). These cells could potentially copurify with HSCs or MSCs and contaminate these cell populations investigated in liver repopulation studies. According

to this hypothesis, rather than being a source of liver-committed SCs, BM could act as a hide out for recirculating pluripotent SCs that might be deposited early during development in BM and could be a source for tissue/organ regeneration (Ratajczak *et al.*, 2004; Kucia *et al.*, 2006). Therefore, the present distinction between HSCs and MSCs may become obsolete, given the heterogeneity and possible overlaps of these various BMSC populations, which could share a common stem cellness core (Piscaglia *et al.*, 2008).

Adipose tissue also has been reported as a rich source of easily accessible MSCs capable of hepatic differentiation *in vitro* and *in vivo* (Zuk *et al.*, 2001; Banas *et al.*, 2007). These are similar to BM-MSCs in terms of surface antigen marker profile and differentiation potential, and display an even higher proliferative capacity *in vitro* (Liu *et al.*, 2007; Kern *et al.*, 2006). Overall, despite an incomplete knowledge of their biological properties, the plasticity and accessibility of HSCs and MSCs from BM and adipose tissue render these adult SCs an attractive tool for the regenerative medicine.

2.3.3.7 Bone Marrow SC Transplantation

BMSCs seem to be physiologically involved in liver repair in humans. A spontaneous mobilization of $CD34^+$ cells has been reported following liver resection in patients with primary liver cancer or metastasis (De Silvestro *et al.*, 2004) Similarly, a significant increase in the percentage of $CD133^+$ cells has been found by Gehling *et al.* in blood samples of healthy living liver donors and further *in vitro* investigations have demonstrated that the mobilized cells were indeed liver committed (Gehling *et al.*, 2005). Recently, the same authors observed that liver cirrhosis is associated with an intermittent mobilization of various populations of liver committed cells of putative BM origin into the circulation (Gehling *et al.*, 2010). The possible therapeutic interest of BMSCs in hepatology was first investigated in 2005: intraportal autologous transplantation of $CD133^+$ BMSCs in patients with liver cancer undergoing portal embolization before extensive liver resection (LR) achieved some clinical improvement (am Esch *et al.*, 2005). Similar results were obtained two years later by Fürst *et al.* (2007), who concluded that in patients with malignant liver lesions a combination of portal vein embolization and $CD133^+$ BMSC administration increased the degree of hepatic regeneration in comparison with embolization alone. Recently, a significant increase of liver

function post LR has been documented in patients with cirrhosis and hepatocellular carcinoma, following autologous BMSC transplantation prior to surgery (Ismail *et al.*, 2010).

2.4 Hemophilia A

Hemophilia A is the most common hemorrhagic disorder, affecting 1 in approximately 5,000 males (Sadler and Davie, 1987). Hemophilia A has been associated with deficiency of a plasma component since 1937, when Patek and Taylor showed that the clotting defect of hemophilic plasma could be corrected by plasma of a normal individual. This component was called antihemophilic factor and later, FVIII. Studies using preparations enriched in FVIII activity have established FVIII as being the cofactor of activated factor IX in the factor X–activating complex of the intrinsic coagulation pathway (Mann *et al.*, 1990). By 1983 FVIII (FVIII), the protein absent or defective in haemophilia A, had been purified to homogeneity (Rotblat *et al* 1983) and shortly afterwards the gene was successfully cloned (Gitschier *et al* 1984).

2.4.1 *FVIII* Gene

In humans, the gene of *FVIII* is located at the tip of the long arm of the X chromosome. It spans over 180 kb, and as such is one of the largest genes known. Its transcription may require several hours assuming a transcription rate of 10 nucleotides per second, and yields a 9 kb mRNA product (Gitschier *et al* 1984). The *FVIII* gene comprises 26 exons, which encode a polypeptide chain of 2351 amino acids. This includes a signal peptide of 19 and a mature protein of 2332 amino acids. Analysis of the deduced primary structure determined from the cloned *FVIII* cDNA showed the presence of a discrete domain structure: A1-a1-A2-a2-B-a3-A3-C1-C2 (Vehar *et al.*, 1984; Toole *et al.*, 1984). The A domains are bordered by short spacers (a1, a2, and a3) that contain clusters of Asp and Glu residues, the so called acidic regions.

DNA clones corresponding in the mouse homolog of the human *FVIII* gene have been isolated and sequenced. The murine gene is expressed in most tissues examined and the mRNA is approximately 1.8 kb smaller than the human transcript, primarily due to a shorter

3′ untranslated region. The mouse cDNA encodes a protein of 2319 amino acids, 32 amino acids shorter than human FVIII, with 74% identity to the human sequence. Further comparison shows that amino acid sequences in the functionally important A and C domains are highly conserved (84-93% identity), whereas the B domains and the two acidic domains are more divergent (42-70% identity). All thrombin/factor Xa cleavage sites and all but one activated protein C cleavage site are conserved in mouse FVIII, as well as a tyrosine residue needed for von Willebrand factor (vWF) binding. These findings suggest that mouse FVIII operates in the clotting cascade much like the human protein, but reveal some differences that may have functional significance (Elder *et al.*, 1993).

2.4.2 Biosynthesis and Secretion of FVIII

The site of the cellular origin for the biosynthesis of FVIII remains controversial despite studies that date back nearly 50 years (Lenting *et al.*, 1998). Several tissues have the potential of expressing the *FVIII* gene. *FVIII* mRNA has been demonstrated in a variety of tissues, including spleen, lymph nodes, liver, and kidney (Levinson *et al.*, 1992, Elder *et al.*, 1993). Human and canine hemophilia A are cured by liver transplantation (Bontempo *et al.*, 1987; Webster *et al*, 1971), which demonstrates that the liver contributes significantly to FVIII synthesis. Hepatocytes (Wion *et al.*, 1985), liver sinusoidal endothelial cells (LSECs) (Stel *et al.*, 1983), or both (Zelechowska *et al.*, 1985), have been proposed as sites of FVIII synthesis. Steady-state levels of *FVIII* mRNA have been reported in hepatocytes and LSECs indicating that both LSECs and hepatocytes synthesize *FVIII* mRNA. It has been observed that LSECs in culture secrete active FVIII (Do *et al.*, 1999) and the promotor region of the *FVIII* gene comprises responsive elements that are characteristic for hepatocyte-specific expression (Figueiredo and Brownlee, 1995). In immuno-ultrastructural studies, FVIII protein was detected in the rough endoplasmatic reticulum and the Golgi apparatus of hepatocytes (Zelechowska *et al.*, 1985).

The initial stage of secretion involves the translocation of the mature 2332 amino acid polypeptide into the lumen of the endoplasmatic reticulum, where N-linked glycosylation occurs (Dorner *et al.*, 1989; Marquette *et al.*, 1995). After transportation to the Golgi apparatus, FVIII is subject to further processing, including modification of the N-linked

oligosaccharides to complex-type structures, O-linked glycosylation, and sulfation of specific Tyr-residues. In addition, FVIII undergoes intracelullar proteolysis leading to disruption of the covalent linkage of the FVIII heavy chain (A1-a1-A2-a2-B) and light chain (a3-A3-C1-C2), giving rise to the heterodimeric molecule that circulates in plasma (Kaufman *et al.*, 1988; Hutton, 1990; Barr, 1991). The FVIII heavy and light chains remain noncovalently associated through the A1 and A3 domain in a metal ion-dependent manner (Fass *et al*, 1982; Fay *et al.*, 1986; Fay and Smudzin, 1992).

2.4.3 Role of FVIII in Blood Coagulation

Immediately after its release into the circulation, the FVIII heterodimer interacts with its carrier protein vWF to form a tight, noncovalent complex. Each monomer of the multimeric

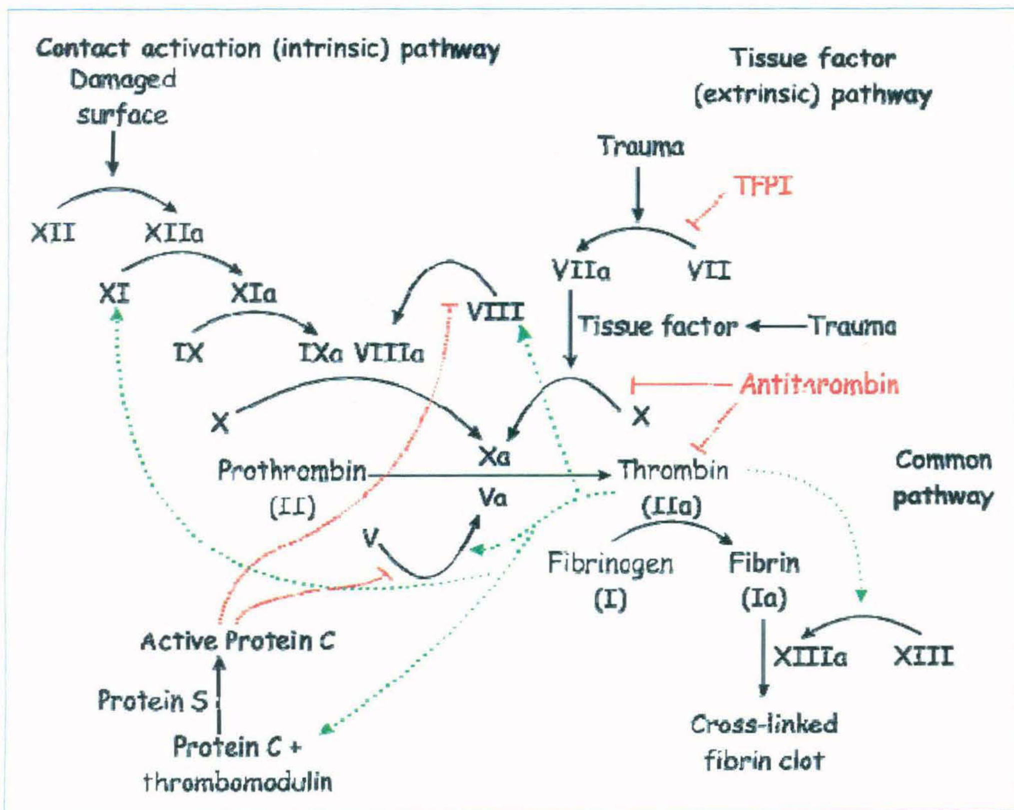


Figure 3.3. Schematic representation of blood coagulation pathways. FVIII acts as a scaffold for the formation of 'tenase complex' which leads to generation of FXa

vWF protein is able to bind one FVIII molecule with high affinity (Lollar *et al.*, 1988; Saenko and Scandella, 1997). During the intrinsic coagulation process, cleavage of FVIII light chain by thrombin or activated factor X (FXa) activates FVIII (FVIIIa) and also results in a 1,600-fold decrease in its affinity for vWF. FVIIIa binds to the cell membrane surface. This subsequently favors binding of activated factor IX (FIXa) to FVIIIa, which is no longer associated with vWF. Binding of FVIIIa markedly enhances the proteolytic activity of FIXa. This sequence of events leads to the assembly of the membrane-bound FVIIIa–FIXa complex that activates factor X and hence the formation of blood clots (Figure 3.3) (Saenko and Scandella, 1997).

2.4.4 Pathogenesis of Hemophilia A

Hemophilia A is an X-linked hereditary bleeding disorder with an incidence of approximately 1/5000 males that results from either reduced or absent levels of coagulation FVIII (White, 1995). Aberrant biosynthesis or secretion may result from several defects. Gross deletions or rearrangements may result in impaired transcription, RNA processing, or translation. Defective secretion may further be caused by apparently minor gene defects like single

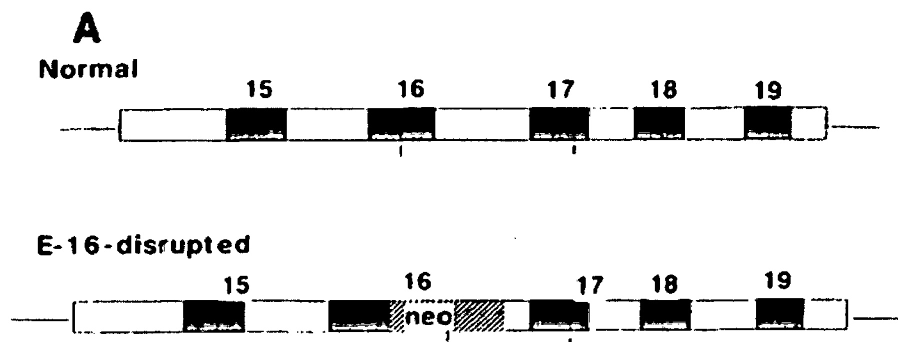


Figure 3.4. Diagram showing the disruption of FVIII gene by insertion of *neo* sequences in to exon 16.

missense mutations (Lenting *et al.*, 1998). Recombinant FVIII mutant proteins expressed in mouse fibroblasts (Voorberg *et al.*, 1996) and COS-1 monkey kidney cells (Pipe and Kaufman, 1996) appear to be functionally normal, but are poorly secreted. Disruption of either exon 16

or 17 of FVIII gene by insertion of a neo cassette resulted in a hemophilic condition in mice. This resembled naturally occurring hemophilia A in that, of all males carrying the mutated X chromosome, 2 out of 3 died due to excessive hemorrhage after experimentally induced injury whereas all the unaffected males and all the females which were heterozygous, survived. These mice could be rescued from excessive hemorrhage by transfusion of plasma from normal mice in a dose dependent manner (Figure 3.4) (Bi *et al.*, 1995).

Hemophilia A exhibit three levels of severity: severe, moderate, and mild. The risk for bleeding is linked to the degree of severity. In severe cases, initial hemorrhaging occurs before the age of 1 year, and in moderate cases bleeding more commonly starts later, i.e. at 1 to 2 years of age. Bleeding is spontaneous or occurs after minimal trauma. In mild hemophilia, bleeding problems are often related to surgery and injuries. In severe and moderate hemophilia, the main symptom is hemorrhaging primarily affecting the ankle, knee and elbow joints, and muscles. Joint hemorrhages lead to chronic joint inflammation and degradation of articular cartilage, leading to stiffness, limitation in movement, and pain resulting in mobility-related disabilities. Patients with hemophilia are also at risk for other severe, sometimes life-threatening types of hemorrhage, *e.g.*, cerebral or gastric hemorrhage.

2.4.5 Treatment Strategies for Hemophilia A

2.4.5.1 Transfusion of blood or plasma

In the 1940s physicians began to treat hemorrhaging with blood transfusions and started using blood plasma in the 1940s and 1950s. However, blood or plasma transfusion alone cannot completely normalize blood clotting since very large volumes of blood or plasma needed to be infused. Treatment improved with access to products that concentrated the coagulation factors two to three times, and later more concentrated products were produced, a prerequisite for effective replacement therapy. However, many hemophilics who received plasma therapy were infected with hepatitis B , hepatitis C or HIV. The problem of plasma-mediated infection was resolved later by improved manufacturing techniques (Swedish Council on Health Technology Assessment, 2011). Treatment of hemophiliacs using

plasma derived FVIII concentrates has been shown to result in more than 82% of the treated population responding well to the treatment with no anti-FVIII antibody [inhibitor] development (Powell *et al.*, 2000; Nemes *et al.*, 2007).

An important step forward for understanding hemophilia and improving treatment was the publication in 1984 regarding sequencing the FVIII gene and the expression and production of human FVIII in cultured mammal cells (Gitschier *et al.*, 1984; Wood *et al.*, 1984). Various studies show the efficiency of treatment of hemophilia A using recombinant FVIII concentrates to be good or excellent. However, formation of inhibitors in the treated population has been shown to range from <1% to >34% (Courter and Bedrosian, 2001; Lusher *et al.*, 2003; Yoshioka *et al.*, 2003, Lusher *et al.*, 2004). High levels of inhibitors neutralize the administered FVIII rendering the treatment ineffective. As a means of overcoming this problem, clinicians usually try to overcome the immune defence by immune tolerance induction (ITI), which means administration of factor concentrate daily at high doses (100–200 IU per kg), which can take 6 to 24 months. Studies on ITI show that a dose dependent response can be achieved (Platokouki *et al.*, 2009; Valentino *et al.*, 2009), with up to 89% patients (Orsini, *et al.*, 2005) developing tolerance in high dose regimens, whereas low dose regimens were not sufficiently effective (Unuvar *et al.*, 2008). Immunotolerance treatment is successful in up to 60–80 percent of the patients, who then become treatable by using standard replacement therapy.

2.4.5.2 Bypass Products

Several studies have demonstrated the effectiveness of the use of bypass products in the treatment of hemophilia A, in which factors or components of the coagulation cascade, other than FVIII were used. Treatment using recombinant FVIIa resulted in a dose dependent response, where high doses at frequent intervals (Shapiro *et al.*, 1998; Shirahata *et al.*, 2001; Ingerslev, 2000) resulted in higher efficiency of treatment. Early treatment (Santagostino *et al.*, 1999; Lusher, 1998) was also found to increase the efficiency. However, the efficiency was less than 100% in most regimens and in some cases, the treatment failed altogether (Santagostino *et al.*, 1999; Lusher, 1998; Shirahata *et al.*, 2001). Use of activated prothrombin complex concentrates (aPCC) was another approach which resulted in effective

hemostasis in 81% of the cases whereas it failed in 1.8% of the cases. Adverse events such as myocardial infarction and disseminated intravascular coagulation were reported in some cases (Negrier *et al.*, 1997). Prophylactic treatment with recombinant FVIIa was found to provide superior results when compared to the aPCC mediated prophylaxis (Young *et al.*, 2008; Jimenez-Yuste *et al.*, 2009)

2.4.5.3 Gene Therapy

Treating hemophilia with gene therapy appears promising because the disease is caused by a single gene defect and because only a small increase (5%) (Gan *et al.*, 2006) in gene product could essentially transform a severe form of hemophilia into a mild one. Another advantage is that although clotting factors are made in the liver, they can be synthesized in a wide variety of cells (High, 2006). With continuous supply of gene product, gene therapy could potentially cure hemophilia (VandenDriessche *et al.*, 2001). Gene therapy has been successful in greatly improving, if not curing hemophilia in dogs (Chuah *et al.*, 2001) and in mice with knock-out mutations for the coagulating factor genes. Genes can be integrated into highly reproducing SCs so that all the daughter cells express the gene and its product. Alternatively, genes can be integrated into long living cells such as skeletal muscle, cardiac muscle and central nervous sytem SCs to ensure continuous expression (High, 2006). For gene delivery into the target cells, viral vectors or naked DNA plasmids (Rick *et al.*, 2003) can be used. The use of HIV mediated transfer of FVIII has been successfully demonstrated in mice such that therapeutic levels of the clotting factor were produced (Kootstra *et al.*, 2003). However, in spite of all the promising results observed in animal models, human subjects have not yet demonstrated the universal success of gene therapy (Manucci, 2003; Pipe, 2004). If cells must be implanted surgically, patients with severe hemophilia would become untreatable. Patients with liver diseases could not use genetically engineered hepatic cells. Production of inhibitors is a major problem, which depends upon the mode of transmission of the target gene, the target cells, the dose of vector and the type of vector (VandenDriessche *et al.*, 2003). Gan *et al.* (2006) suggested that introduction of gene therapy to newborn animals may prevent the formation of inhibitors, which may not be practical in human patients. Genetic mutation resulting from gene integration in to target cells has led to formation of neoplasms in human patients, and this necessitates further measures to ensure

the safety of the patients (Mannucci, 2003; High, 2006). Consequent to all these drawbacks, gene therapy is still in developmental stages and is very expensive and require strict supervision.

2.5 Allogeneic Transplantation

2.5.1 Transplantation of Hematopoietic Cells

Allogeneic hematopoietic transplantation became feasible in the early 1960s, after the identification and typing of human leukocyte antigens (HLAs). The first transplants from HLA matched siblings were performed in 1968 in two children with congenital immunodeficiency, and these transplants were successful because the recipients could not reject the allograft. However, in the current setting, 75% of patients do not have an HLA identical sibling because, as the genes for HLA are closely linked on chromosome 6 and are inherited as haplotypes, two siblings have one chance in four of being HLA identical (Ruggeri *et al.*, 2006). As a result, allograft rejection has been the most important hurdle in successful transplantation of organs or cells.

2.5.2 Immune Activation and Regulation

The immune reaction against donor tissue (antigens), also called the alloresponse, is the driving force of graft rejection. Rejection is an inflammatory reaction in the donor organ that involves different cell types. Alloreactive T cells play a key role in the immune reaction and can be subdivided into two lineages; the helper T cells ($CD4^+$) and the cytotoxic T cells ($CD8^+$). $CD4^+$ T cells are known for their central role in the initiation of an immune response and their ability to coordinate the differentiation and effector function of other immune cells, while $CD8^+$ T cells are characterized by their cytotoxic activity.

The activation of T cells depends on a cascade of signals. First the interaction of the T cell and the antigen presenting cell: the T cell receptor (TCR) binds to a self- major histocompatibility complex (MHC) on an antigen presenting cell. When TCR triggering is accompanied by a co-stimulatory signal from the antigen presenting cell (APC), cytokine genes are activated

causing production and release of cytokines, an essential element in the course of immune activation. IL-2 is one of the most important activating cytokines causing activation, proliferation and differentiation of T cells. Once adequate activation has occurred, T cells migrate to the donor organ and mediate several effector mechanisms, such as cytotoxicity, delayed-type hypersensitivity and antibody production. These effector mechanisms damage the donor organ and are recognized as acute rejection (Le Moine *et al.*, 2002).

Despite the restriction between self-MHC and TCR, as many as 10% of a donor or host's T cell population can specifically recognize allogeneic MHC molecules (Lindahl and Wilson, 1977; Suchin *et al.*, 2001), resulting in the apparent paradox of T cell allorecognition. This may be due to the T cells recognizing thousands of antigenic determinants per cell in the form of the polymorphisms within the foreign MHC molecule itself (Bevan, 1984), or because the allo-MHC molecule is similar enough to the self-MHC molecule for the alloreactive TCR to bind to it and recognize the presented peptide as foreign (Matzinger and Bevan, 1977).

2.5.3 Effector Mechanisms

The final common pathway for the cytolytic processes is triggering of apoptosis in the target cell (Krupnick *et al.*, 2002). Cytotoxic T lymphocytes (CTL) (which include $CD8^+$ and some $CD4^+$ T cells) and natural killer (NK) cells are involved in the elimination of infected and foreign cells by the immune system. $CD8^+$ and $CD4^+$ T cells are present in acutely rejecting allografts along with a small number of NK cells (Hanson *et al.*, 1988; Trentin *et al.*, 1992). $CD8^+$ T cells are the best understood CTL population. Resting $CD8^+$ T cells differentiate into CTLs after an encounter with foreign peptide–MHC class I in the context of other activating signals (Parish and Kaech, 2009). These CTLs then migrate to sites of immune activation and induce the death of target cells. Memory $CD8^+$ T cells may be one of the earliest leukocytes to enter allografts and trigger the rejection process (Schenk *et al.*, 2008). $CD8^+$ T cell deficiency prevents the acute rejection of heart allografts in a minor histocompatibility antigen mismatched mouse model (Youssef *et al.*, 2004). Vascular endothelial cells activate allogeneic T cell responses and are also an important target of CTLs in all solid organ allografts (Choy *et al.*, 2007).

After activation of the CTLs, they form cytotoxic granules that contain perforin and granzymes (Krupnick *et al.*, 2002). At the time of target cell identification and engagement, these granules fuse with the effector cell membrane and extrude the content into the immunological synapse. Perforin facilitates the entry of granzymes into the target cell cytoplasm where granzyme B can trigger apoptosis through several different mechanisms, including direct cleavage of procaspase-3 and indirect activation of procaspase-9 (Voskoboinik *et al.*, 2006). This has been shown to play the dominant role in apoptosis induction in allograft rejection. Alternatively, $CD8^+$ CTLs can also use the Fas-dependent pathway to induce cytolysis and apoptosis. The Fas pathway is also important in limiting T cell proliferation in response to antigenic stimulation; this is known as fratricide between activated CTLs. Cell-mediated cytotoxicity has been shown to play an important role in acute, although not chronic, allograft rejection which is also termed as host versus graft disease (HVGD).

The NK cells are important in transplantation because of their ability to distinguish allogeneic cells from self and their potent cytolytic effector mechanisms (Kitchens *et al.*, 2006). These cells can mount a maximal effector response without any prior immune sensitization. Unlike T and B cells, NK cells are activated by the absence of MHC molecules on the surface of target cells according to the 'missing self' hypothesis. The recognition is mediated by various NK inhibitory receptors triggered by specific alleles of MHC class I antigens on cell surfaces. In addition, they also possess stimulatory receptors, which are triggered by antigens on non-self cells (Joncker and Raulet, 2008). NK cells also provide help to CD28-positive host T cells, thereby promoting allograft rejection (McNerney *et al.*, 2006). NK cells are now being recognized as active participants in the acute and chronic rejection of solid tissue grafts. Recent studies have indicated that NK cells are present and activated following infiltration into solid organ allografts (Kitchens *et al.*, 2006).

2.5.4 Graft Versus Host Disease

Graft versus host disease (GVHD) is a complex disease resulting from donor T cell recognition of a genetically disparate recipient that is unable to reject donor cells after allogeneic HSC transplantation. The classical scheme of GVHD (Welniak *et al.*, 2007; Ferrara *et al.*, 2009)

development includes alloreactive T cell activation, costimulation, expansion and differentiation; activated T cell migration to GVHD target tissues such as gut, liver, skin, and lung followed by the recruitment of other effector leukocytes (Wysocki *et al.*, 2005); and destruction of the target tissues by effector T cells. Destruction occurs via exposure to cell surface and release of soluble immune effector molecules.

2.5.4.1 Pathogenesis of GVHD

Recent advances have indicated the presence of a subset of postmitotic, self-renewing $CD44^{lo}/CD62L^{hi}/CD8^{+}$ T cells that can generate and sustain all allogeneic T cell subsets in GVHD reactions, including central memory, effector memory, and effector $CD8^{+}$ T cells (Zhang *et al.*, 2005a). In mouse models, , $CD4^{+}$ T cells induce acute GVHD to MHC class II differences and $CD8^{+}$ T cells induce acute disease to MHC class I differences. Murine studies with minor histocompatibility antigen (MiHA)–disparate models have demonstrated that GVHD initiation requires donor T cell recognition of host antigen in the context of host APCs (Hadeiba *et al.*, 2008). Subsequently donor-derived APCs are able to augment $CD8^{+}$ T cell–mediated GVHD by acquiring and presenting host antigens (Matte *et al.*, 2004).

Major roles for GVHD initiation has been ascribed to a positive (CD28/B7) and an inhibitory (CTLA-4:B7) pathway. In response to tissue injury and activated T cells, inhibitory pathways are up-regulated in an attempt to protect the host against injury. CTLA-4 and programmed death-1 (CD279) are upregulated on donor T cells during acute GVHD and serve to dampen the immune response. Both also are primarily expressed in the cytoplasm of activated T cells and $CD4^{+}CD25^{+}$ regulatory T cells.

2.5.4.2 T-cell Subpopulations Involved in GVHD

2.5.4.2.1 Naive T Cells

Using new methods in rodents including green fluorescent protein marking or bioluminescence technology, it has been reported that T cells can undergo a massive and much earlier than previously thought expansion in lymph nodes and Peyer patches (Beilhack

et al., 2005). In mice, naive $CD44^{lo}CD62L^{hi}$ $CD8^{+}$ T cells generate and sustain allogeneic $CD8^{+}$ T cells in GVHD reactions (Anderson *et al.*, 2003; 2004). Alloantigen sensitized effector memory $CD44^{hi}CD62L^{lo}$ as well as naive phenotype $CD44^{lo}CD62L^{hi}$ T cells have been found to be capable of causing GVHD after adoptive transfer into secondary recipients (Zhang *et al.*, 2005b). Researchers have considered acute GVHD to be a T_h1/Tcytotoxic type disease on the basis of the predominance of cytotoxic T cell mediated pathology and of increased production of T_h1-type cytokines (Nikolic *et al.*, 2000) that is detectable in the serum or donor T cells during acute GVHD.

2.5.4.2.2 Regulatory T Cells

$CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells (T_{reg}s) have potent suppressor activity both *in vitro* and *in vivo*. Donor T_{reg} infusion blocks acute GVHD. Murine L-selectin (CD62L)–expressing T_{reg}s preferentially home to secondary lymphoid organs, and in particular lymph nodes, resulting in GVHD prevention (Ermann *et al.*, 2005). Conversely, depletion of $CD25^{+}$ T cells from the donor graft or in the recipient immediately after allogeneic HSC transplantation promotes acute and chronic GVHD in various mouse models (Muriglan *et al.*, 2004; Hoffmann *et al.*, 2002).

2.5.4.2.3 NKT Cells

A second inhibitory population shown to inhibit acute GVHD lethality is the natural killer T (NKT) subset that coexpresses NK and T cell surface determinants (Zeng *et al.*, 1999). In rodents, total lymphoid irradiation combined with antithymocyte globulin has been shown to induce host NKT cells that also promote the generation of T_{reg}s and the production and release of anti-inflammatory cytokines (Kohrt *et al.*, 2009).

2.5.4.3 T-cell Trafficking

During a GVHD reaction, donor T cells initially migrate to spleen and peripheral lymphoid tissues within hours (Panoskaltsis-Mortari *et al.*, 2004), receives activation signals by APCs, and then subsequently migrate to specific GVHD target organ sites (Chakraverty *et al.*, 2006),

After migration of alloreactive effector T cells to the target tissues of GVHD, these cells can mediate tissue destruction through both direct cytotoxic activity and the recruitment of other leukocytes.

2.5.5 Prevention of Immune Rejection

There are two main strategies for preventing rejection of the allograft: immunosuppressive therapy, and tolerance induction. Without either intervention, transplanted organs or tissues are rapidly rejected by the recipient's immune system.

2.5.5.1 Immunosuppressive Drugs

Immunotherapy has been a feature of the transplant arena since the 1950s. The current immunosuppressive drugs effectively reduce the immune response to alloantigens, resulting in a relatively low incidence of acute rejection. Most immunosuppressive drugs target the intracellular signals involved in T cell activation following antigen presentation (Denton *et al.*, 1999; Taylor *et al.*, 2005). Conventional immunosuppressive drugs include corticosteroids, calcineurin inhibitors, IL-2 receptor-blocking antibodies, rapamycin and mycophenolate mofetil. The long-term administration of immunosuppressants leads to many debilitating side effects influencing patient- and graft survival (Lopez *et al.*, 2006). Therefore, it is essential to minimize the use of immunosuppression.

There are three distinctive eras of immunosuppressive drugs (Cendales and Hardy, 2000). The first era extended from 1956 to 1984, starting with the use of total body irradiation, steroids, and then azathioprine, as well as initial use of polyclonal anti-lymphocyte antibodies. With the introduction of cyclosporine A in 1984, the transplantation field took a giant leap forward and was associated with the initial use of monoclonal antibodies, particularly Muromonab-CD3 (OKT3) (Benfield *et al.*, 2005). From 1994, there was a widespread proliferation of newer immunosuppressive drugs, particularly monoclonal antibodies directed against the IL-2 receptor and various T cell receptors. In addition, newer synergistic drugs targeting the signaling between cells—*e.g.* Tac and sirolimus—and anti-proliferative drugs—*e.g.* mycophenolate—were increasingly being used. All agents currently

used in the mainstay of immunosuppressive regimens to prevent rejections (except corticosteroids) interfere with discrete sites in the T and B cell activation and migration cascade.

2.5.5.1.1 Induction therapy

The goal of induction therapy is to turn off the recipient immune system in anticipation of the allograft. These drugs are very potent and induce profound T cell depletion before allotransplantation, with gradual posttransplant T cell repopulation, reduce the risk of acute rejection, help to decrease the dose of maintenance immunosuppressive drugs, and facilitate delayed introduction of calcineurin inhibitors (Starzl *et al.*, 1998; Starzl and Zinkernagel, 2001). Anti-thymocyte globulin (ATG) is a polyclonal antibody preparation of purified immunoglobulin from animals - rabbits or horses - after immunization with human lymphocytes (Halloran, 2004). These polyclonal antibodies opsonize and deplete T cells by causing complement-mediated lysis or reticuloendothelial cell-mediated phagocytosis (Starzl *et al.*, 1967). Use of ATG as an induction agent along with other immunosuppressive drugs has been shown to result in a significant reduction in acute rejection rates for various solid organs (Charpentier *et al.*, 2003; Eason *et al.*, 2003; Reyes *et al.*, 2005). Although polyclonal antibodies are inexpensive to produce, they have a low degree of specificity to the immunizing antigen. Their side effects include formation of anti-antibodies, serum sickness, dose-limiting leukopenia, and infusion-related reactions such as fever, chills, and other systemic effects (Gaber *et al.*, 1998). Daclizumab and Basiliximab are humanized and chimerical immunoglobin G (IgG) monoclonal antibodies with a high affinity for the alpha subunit of the IL-2 receptor (CD25). These agents inhibit the binding of IL-2 to IL-2 receptor on T cells, thereby competitively inhibiting the activation and proliferation of T cells. They have been used as induction agents in various protocols for solid organ transplantation (Chapman and Keating, 2003; Vincenti *et al.*, 1998). Alemtuzumab (campath-1H) is a humanized monoclonal antibody against membrane glycoprotein CD52 that is present on T cells, B cells, monocytes, macrophages, natural killer cells, and granulocytes (Ciancio *et al.*, 2004). Alemtuzumab rapidly depletes CD52 expressing cells both centrally and peripherally (Kirk *et al.*, 2003).

2.5.5.1.2 Maintenance Therapy

The highest risk of rejection occurs during the first few months post-transplant, necessitating potent immunosuppressants as an induction. The goal of maintenance therapy is to use minimal immunosuppression—just enough to prevent rejection while keeping the host immunocompetent to infectious diseases and tumor surveillance. Multiple immunosuppressants targeting the immune system at different levels are used at a low dose to produce a synergistic effect with minimal toxicity.

2.5.5.1.3 Corticosteroids

Corticosteroids are pleiotropic hormones that are used for their potent anti-inflammatory and immunomodulatory action. Corticosteroids have been the mainstay of immunosuppression since the 1920s. Prednisone has been used as an integral part of maintenance therapy for most solid organ transplantations. High doses of methyprednisone have been used successfully to treat acute rejections following transplantation. Corticosteroids interrupt the immune system at various stages due to universal expression of glucocorticoid receptor. They have a negative effect on peripheral blood lymphocyte and monocyte count (Fauci and Dale, 1974; Rinehart *et al.*, 1974). In addition, they inhibit T cell activation, pro-inflammatory cytokine production, and prostaglandin production (Claman, 1983). Corticoteroids inhibit the action of transcription factors, like NF-κB and AP-1 that are involved in transcription of many cytokine and chemokine genes including IL-2, TNF-α and IFN-γ. It is known that $CD4^+CD25^+Foxp3^+$ T cells highly express the glucocorticoid receptor as well as glucocorticoid-induced TNF receptor (GITR), a potent T cell co-stimulatory receptor and regulator of T_{reg} function (Chen *et al.*, 2004). The activity of GITR has been implicated in peripheral tolerance since inhibition or a deficiency of GITR increases T cell proliferation by abrogating the suppressive function of T_{reg} (Shimizu *et al.*, 2002; McHugh *et al.*, 2002; Ronchetti *et al.*, 2002; Kanamaru *et al.*, 2004). The first direct evidence that steroids affect T_{reg} came from the observation that the female sex hormone, estrogen, upregulates forkhead box P3 (Foxp3) expression in mice, both *in vitro* and *in vivo* (Polanczyk *et al.*, 2004). Similar results were reported for the synthetic corticosteroid, dexamethasone, which

induced Foxp3 expression in short and long-term T cell cultures, while preserving the suppressive capacity of T_{reg} (Karagiannidis *et al.*, 2004; Dao *et al.*, 2004). Furthermore, $CD4^{+}CD25^{+}$ T cells seemed resistant to dexamethasone-induced T cell apoptosis (Chen *et al.*, 2004). In mice, short-term simultaneous administration of dexamethasone and IL-2 expanded $Foxp3^{+}$ T_{reg} in peripheral lymphoid tissues (Chen *et al.*, 2006). This treatment improved the suppressive capacity of splenic T_{reg} in such a way that they were able to prevent the onset of autoimmune disease. Taken together, indicate that corticosteroids improve the survival and function of T_{reg}. It is possible that this may account for some of the anti-inflammatory and immunosuppressive efficacy of steroids. However, long-term complications such as delayed wound healing, opportunistic infections, metabolic derangements, and avascular necrosis of the hip warrant judicious use of steroids as maintenance therapy.

2.5.5.1.4 Calcineurin Inhibitors

Calcineurin is an important protein in the intracellular signaling cascade for cytokine production. Calcineurin inhibitors downregulate cytokine production without turning it off completely. With the introduction of Cyclosporin A in the 1980s, transplantation became the preferred treatment for end-stage organ disease (Starzl *et al.*, 1981). In combination with steroids, Cyclosporin A dramatically reduces acute rejection rates post-transplant. However, Cyclosporin A causes dose related nephrotoxicity. In addition, hepatotoxicity, neurotoxicity, hypertension, and gingival hyperplasia have limited the use of Cyclosporin A.

Tacrolimus is a macrolide antibiotic produced by *Streptococcus tsukubaensis*. It is 10–200 times more potent than Cyclosporin A (Pirsch *et al.*, 1997). It binds to the intracellular FK506 binding protein (FKBP-12) and blocks calcineurin mediated cytokine production. It has become a standard immunosuppressant for organ transplantation. Due to its potency, tacrolimus has been used as monotherapy for kidney transplantation (Tan *et al.*, 2006a; Shapiro *et al.*, 2006). However, nephrotoxicity, neurotoxicity, and inability to prevent chronic rejection have led to a search for newer immunosuppressants.

2.5.5.1.5 Other Immunosuppressive Drugs

Sirolimus (rapamycin) is an antibiotic isolated from *Streptococcus hygroscopicus*. It binds to FKBP-12 and inhibits mTOR, a serine-threonine protein kinase, thereby preventing cell cycle progression from the G1 to the S phase. It has been shown to induce Foxp3$^+$ T_{reg} cells (Louis *et al.*, 2006). Its major adverse effects include delayed wound healing, hyperlipidemia, anemia, and thrombocytopenia.

Mycophenolate mofetil is a reversible, non-competitive inhibitor of inosine monophosphate dehydrogenase-2, which is required for DNA synthesis in proliferating T- and B-cells. It thus blocks proliferation of T cells and suppresses B-cell antibody production without affecting BM and parenchymal cells (Allison and Eugui, 1993). It has been shown to delay the onset of chronic rejection (Shimizu *et al.*, 2004). In combination with sirolimus, it has been used in a hand transplants patient to prevent anticipated chronic rejection (Schneeberger *et al.*, 2006). Its side effects are mild and include leukopenia, gastritis, and opportunistic infections with cytomegalovirus.

Advances in transplantation pharmacology have led to an explosion in immunosuppressant drug design, with newer drugs specifically designed to inhibit selective aspects of the immune system while at the same time minimizing toxicity. Everolimus is a macrolide antibiotic similar to sirolimus, but with a better oral bioavailability profile and efficacy. It has been approved by US FDA for use in solid organ transplantation (Novartis, 2010). Fingolimod (FTY720) is a sphingosine-1-phosphate receptor agonist that prevents egression of lymphocytes from secondary lymphoid tissue and prevents CD4$^+$, CD8$^+$, and memory T cells from accessing inflammation sites (Zhang *et al.*, 2006). Costimulation blockade has also been trialled successfully in animal models of organ transplantation, but has not replicated these results in human studies.

2.5.5.2 Establishment of Chimerism

One of the best-studied approaches for establishing tolerance is HSC chimerism. In chimerism, tissues from two genetically distinct organisms co-exist in one organism. Two

types of chimerism have been described: macrochimerism and microchimerism. Macrochimerism usually occurs when BM is transplanted in a conditioned recipient. The donor HSCs engraft in the recipient and produce all its lineages, including the donor immune cells. A new hybrid immune system is established in the recipient and reciprocal bidirectional donor:host tolerance results. As low as 1% donor chimerism is sufficient to induce robust tolerance to donor-specific organs, cells, and tissues (Ildstad and Sachs, 1984). Microchimerism arises as a result of migration of passenger leukocytes from a transplanted allograft into an unconditioned recipient. Passenger leukocytes from the transplanted allograft interact with the recipient leukocytes and are hypothesized to lead to clonal exhaustion, resulting in donor-specific tolerance (Burlingham, 1996). In microchimerism, donor HSCs do not engraft, but alternatively hematopoietic-derived cells from the donor organ are produced and migrate systemically. Consequently, not all stem-cell derived lineages are produced and very low levels of donor cells are found in the recipient's blood. Microchimerism has been demonstrated in liver and kidney transplant recipients. Starzl and Zinkernagel (2001) make a strong argument that microchimerism is essential for the maintenance of clonal exhaustion-deletion that is induced by the initial flood of passenger leukocytes during the first few weeks after transplantation. This view is supported by the observation that some transplant recipients require significantly reduced levels or no immunosuppression over time.

In an alternate situation known as mixed chimerism, the donor and recipient hematopoietic systems coexist. Mixed chimerism is associated with donor-specific transplantation tolerance *in vivo* and *in vitro* (Ildstad and Sachs, 1984) and has been shown to effectively induce donor-specific tolerance to a variety of allografts. In humans, BMT induced mixed chimerism has been shown to confer acceptance of donor-specific skin (Mache *et al.*, 2006) and kidney allografts (Trivedi *et al.*, 2005) without long term immunosuppression. An additional advantage is that mixed chimerism prevents chronic rejection (Gammie *et al.*, 1998), the major cause of late graft loss. Mixed chimerism is associated with a lower incidence and severity of GVHD. It retains immunocompetence for primary immune responses (Ruedi *et al.*, 1989) and it can be induced through nonmyeloablative conditioning.

The classic observations of Billingham *et al* in 1953, that H-2 disparate donor BM-derived cells could bring about specific acquired tolerance to skin allografts in fetal or newborn murine recipients, laid the foundations for the goal of establishing donor-specific tolerance in human organ transplantation. By the late 1970s it was observed that multiple nonspecific and subsequently donor-specific blood transfusions in humans often led to an improved allograft acceptance in kidney transplant recipients (Opelz and Terasaki, 1978; Salvatierra *et al.*, 1980). Post- or peri-transplant infusion of donor BM cells together with T cell depletion prolonged and sometimes brought about indefinite allograft survival in adult murine, canine and primate recipients in the absence of chronic immunosuppression (Monaco and Wood, 1970; Hartner *et al.*, 1986; Thomas *et al.*, 1983). These observations have for the most part been associated with the development of chimerism (Wood and Sachs, 1996). Later studies revealed direct evidence linking increased chimerism in the BM compartment with the absence of graft loss and *in vitro* evidence of donor-specific unresponsiveness in human organ transplant recipients (Garcia-Morales, *et al.*, 1997; Garcia-Morales *et al.*, 1998; Ciancio *et al.*, 2001; Mathew *et al.*, 2000)

Early studies demonstrated that transplantation of BM after lethal radiation exposure could cure mice of radiation sickness (Lorenz, *et al.*, 1952). Since then, clinical and experimental data on the use of donor BMT following myelo- or lymphoablative conditioning have shown the ability to induce hematopoietic chimerism and graft tolerance in recipients of solid organ transplantation. This enabled the reduction of and complete weaning from immunosuppression (Scandling *et al.*, 2008; Sykes, 2007). Trials using high doses of donor $CD34^+$ HSC with minimal or non-ablative recipient conditioning showed successful engraftment, reduced adverse events, immunomodulation, and increased allograft survival (Ramshaw *et al.*, 1995; Quesenberry *et al.*, 1997; Ricordi *et al.*, 1997).

The infused donor BM cells or leukocytes that migrate out of the transplanted organ in the nonimmunosuppressed murine recipients first circulate through the blood stream and rapidly disappear (Larsen *et al.*, 1990). It is presumed that the chimeric donor cells home into various tissues where they take up residence and/or multiply depending on the cell type and the microenvironment (Miller *et al.*, 1999; Starzl *et al.*, 1994).

The chimeric cells that have been identified in human organ transplant recipients so far belong to a number of lineages including the SCs, dendritic cells, myeloid precursors and various lymphoid subpopulations (Garcia-Morales *et al.*, 1998; Miller *et al.*, 1999). Starzl *et al.* (1993) described the presence of cells of dendritic morphology in the lymph nodes, skin and blood of organ transplant recipients. Rugeles, *et al.* (1997) further demonstrated that chimeric donor cells included lymphoid (T, B and NK) cells and macrophages. The number of donor cells was found to gradually increase in the BM with a concomitant decrease in the peripheral blood (Ciancio *et al.*, 2001).

2.5.5.3 Invoking Immune Tolerance

Since the early days of organ transplantation, the immune response against the donor organ has been a major topic of transplantation research. The long-term administration of immunosuppressants leads to many debilitating side effects influencing patient- and graft survival (Lopez *et al.*, 2006). Therefore, it is essential to minimize the use of immunosuppression. To accomplish this, a state of immunological non-responsiveness against the donor antigens, also referred to as transplant tolerance, have to be achieved in recipients. A possible strategy that is now extensively investigated is the induction and maintenance of transplant tolerance by T cells. The T cell populations that prevent autoimmunity and maintain immune homeostasis in the host needs to be adapted to this purpose.

There are two types of mechanisms, recessive and dominant, for achieving self-tolerance and immune homeostasis. In the recessive mechanisms of self-tolerance, the fate of antigen exposed self-reactive lymphocytes is determined in a cell-intrinsic manner as happens during thymic selection of lymphocytes or induction of anergy in a lymphocyte after exposure to self antigen. In the dominant or cell-extrinsic mechanism, T_{reg}s actively keep in check the activation and expansion of aberrant or over-reactive lymphocytes, in particular other types of T cells. Disruption in the development or function of T_{reg}s is a primary cause of autoimmune and inflammatory diseases in humans and animals. Moreover, every adaptive immune response involves recruitment and activation of not only effector T and B cells but also T_{reg}s, and that the balance between the two populations is critical for the proper control

of the quality and magnitude of adaptive immune responses and for establishing or breaching tolerance to self- and non-self-antigens (Sakaguchi *et al.*, 2008).

The involvement of T cells in immunoregulation was first realized when adult thymectomy of selected strains of normal rats followed by several rounds of sublethal X-irradiation produces autoimmune thyroiditis and type I diabetes. Importantly, inoculation of normal T cells, in particular $CD4^+$ T cells or $CD4^+CD8^-$ thymocytes, from untreated syngeneic animals inhibits the development of autoimmunity (Sakaguchi *et al.*, 1982; Fowell and Mason, 1993). Efforts to search for a more specific marker with which to delineate the putative autoimmune-preventive $CD4^+$ T cells revealed the CD25 molecule (the IL-2 receptor α chain) as a candidate (Sakaguchi *et al.*, 1995). Transfer of T cell suspensions depleted of $CD25^+$ T cells indeed produces autoimmune disease in athymic nude mice, whereas cotransfer of a small number of $CD25^+CD4^+$ T cells clearly inhibits the development of autoimmunity. T_{reg} depletion produces inflammatory bowel disease, which likely results from excessive immune responses to commensal bacteria in the intestine (Singh *et al.*, 2001). Experimental transplantation models have shown an important role for T_{reg}s in the induction and maintenance of transplant tolerance by suppressing allo-reactive T cells (Wood *et al.*, 2003). Removal or reduction of $CD25^+CD4^+$ T_{reg}s also provokes effective tumor immunity in otherwise nonresponding animals and augments microbial immunity in chronic infection, leading to eradication of tumors or microbes, respectively (Wang and Wang, 2007; Belkaid and Rouse, 2005). $CD25^+CD4^+$ T cells enriched from normal mice suppress allergy, establish tolerance to organ grafts, prevent GVHD after BM transplantation, and promote feto-maternal tolerance (Sakaguchi, 2005). With these findings, it is possible to deduce that the normal immune system generates $CD25^+CD4^+$ T_{reg}s that are engaged in suppressing immune responses toward self, quasi-self such as autologous tumor cells, and non-self such as microbes and allograft. Extensive research has also revealed several other populations of regulatory T cells including $CD4^+$ Tcells, $CD8^+$ T cells (Zhou *et al.*, 2001; Gilliet and Liu, 2002), $CD8^+CD28^-$ T cells (Ciubotariu *et al.*, 1998), NKT cells (Moodycliffe *et al.*, 2000; Seino *et al.*, 2001) and $CD4^-CD8^-$ double negative T cells (Zhang *et al.*, 2000).

Naturally occurring T_{reg}s specifically express the transcription factor Foxp3 (forkhead box P3), a member of the forkhead/ winged-helix family of transcription factors. Foxp3 is a master

regulator of T_{reg} development and function. Foxp3 gene was first identified as the defective gene in the mouse strain Scurfy. Scurfy is an X-linked recessive mutant that is lethal in hemizygous males within a month after birth, exhibiting hyperactivation of $CD4^+$ T cells and overproduction of proinflammatory cytokines (Brunkow *et al.*, 2001). Inoculation of $CD25^+CD4^+$ T cells from normal mice prevents severe systemic inflammation in Scurfy mice (Fontenot *et al.*, 2003). Mutations of the human gene *FOXP3* are the cause of the genetic disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is the human counterpart of Scurfy (Ochs *et al.*, 2005)

IL-2 is another molecule critical for the function of T_{reg}s. The T_{reg} marker CD25 is a component of the high affinity IL-2 receptor and is functionally essential for T_{reg} development. The number of $Foxp3^+$ T_{reg}s is reduced in mice lacking either CD25 or IL-2 (Antony *et al.*, 2006) and T cell-specific deficiency of STAT5a and STAT5b, which mediate signaling from the IL-2R β chain to the nucleus, abrogates the development of $Foxp3^+$ T_{reg}s, producing autoimmune/inflammatory disease (Burchill *et al.*, 2007; Yao *et al.*, 2007). Administration of a high dose of neutralizing anti-IL-2 monoclonal antibody to normal neonatal mice substantially reduces the number of $Foxp3^+CD25^+CD4^+$ T cells and elicits autoimmune diseases similar to those produced by T_{reg} depletion (Setoguchi *et al.*, 2005). In addition, IL-2 is required for sustained expression of Foxp3 and CD25 in natural T_{reg}s and enhances their suppressive function, at least *in vitro* (Fontenot *et al.*, 2005; Shevach *et al.*, 2006). $Foxp3^+$ natural T_{reg}s are already functionally mature and antigen primed in the thymus, before encountering antigen in the periphery (Sakaguchi *et al.*, 1982; Itoh *et al.*, 1999). Whereas naive T cells in the periphery have been shown to acquire Foxp3 expression and consequently T_{reg} function in several experimental settings *in vitro* (Kretschmer, 2005; Chen *et al.*, 2003). Antigen-specific T_{reg}s migrate to and become activated in regional lymph nodes where tissue-specific self-antigens or microbial antigens are presented (Scheinecker *et al.*, 2002; Samy *et al.*, 2005). They also migrate into inflamed tissues, infectious sites, and tumors (Belkaid *et al.*, 2002). Upon antigen exposure in the regional lymph nodes, $Foxp3^+$ T_{reg}s become activated and exert suppression at a much lower concentration of antigen than naive T cells. Once activated by a particular antigen, T_{reg}s can suppress responder T cells irrespective of whether they share antigen specificity with the T_{reg} (Takahashi *et al.*, 1998).

Several mechanisms of T_{reg}-mediated suppression have been proposed, and these include secretion by the T_{reg} of immunosuppressive cytokines like IL-10, TGF-β (Read *et al.*, 2000) or IL-35 (Collison *et al.*, 2007), cell-contact-dependent suppression (Tang and Bluestone, 2008), and functional modification or killing of APC. Alternatively, absorption of cytokines by T_{reg}s may induce apoptosis in responder T cells (Pandiyan *et al.*, 2007).

Transplant tolerance, the ultimate goal in solid organ transplantation, occurs more often after transplantation of the liver compared to other organs. Cessation of immune suppressive therapy without allograft rejection has been reported to be successful in a considerable proportion of liver transplant recipients (Ramos *et al.*, 1995; Devlin *et al.*, 1998). Among the recipients, 10 to 30 percent have been shown to be tolerant and showed no signs of rejection after mmunosuppression withdrawal (Takatsuki *et al.*, 2001; Mazariegos *et al.*, 1997; Girlanda *et al.*, 2005). Animal experiments suggest a possible role for regulatory T cells in tolerance of liver allografts (Hara *et al.*, 2001; Gregori *et al.*, 2001). The observation that operationally tolerant liver transplant recipients display a significantly increased proportion of $CD4^{+}CD25^{hi}$ T cells compared to recipients on immunosuppression, may support this suggestion (Li *et al.*, 2004; Martinez-Llordella *et al.*, 2007).

$Foxp3^{+}$ natural T_{reg}s retain their suppressive function after expansion *in vivo* and *in vitro.* By exploiting this stable suppressive activity and proliferative capacity, strategies that clonally expand antigen-specific natural T_{reg}s while inhibiting the activation and expansion of effector T cells will help to induce transplantation tolerance and suppress graft rejection. Furthermore, in the presence of T_{reg}s that actively maintain graft tolerance, naive T cells could be newly recruited to the graft site and could differentiate into graft specific T_{reg}s, thereby augmenting graft tolerance (Waldmann *et al.*, 2006). Recent report from the hand transplant experience has shown that $Foxp3^{+}$ T_{reg} infiltrate the skin of hand transplant recipients (Eljaafari *et al.*, 2006). This may explain why rejection has not been a major limitation for this highly antigenic tissue burden.

Aims and Objectives

Based on the literature review, the following objectives were undertaken in this study:

1. Studying the engraftment potentials of bone marrow cells transplanted in syngeneic and allogeneic recipient liver of hemophilia A mice.

This study was aimed at understanding the engraftability of undifferentiated bone marrow cells in MHC- mismatched recipient mouse liver. It was planned to create an environment flouring hepatic differentiation of bone marrow progenitor cells in the recipient mice. Analysis of recipient mouse liver for donor derived hepatocytes and their functional competence was to be performed. The usefulness of functional hepatocytes derived from donor cells in curing a hepatocyte associated deficiency disorder was to be studied. This study was to be carried out under syngeneic transplantation set up as well in order to facilitate determination of the clinical usefulness of allogeneic stem cell therapy. It was also planned to compare the magnitude of coagulation factor VIII production in donor derived hepatocytes and liver sinusoidal endothelial cells.

2. Comparison of functional modulation of recipient liver achieved by transplantation of allogeneic bone marrow cells in conjunction with immunomodulation.

It was planned to explore the effect of immunomodulatory techniques in improving the engraftability of allogeneic bone marrow cells in recipient mouse liver and the extent of functional modulation due to them. Comparison of three different methods used to achieve graft acceptance was envisaged:

a. Reconstitution of the recipient mouse hematopoietic system by establishing donor-specific bone marrow chimerism.

b. Immunosuppression of recipient mouse by use of dexamethasone.

c. Induction of donor-specific immune tolerance in the recipient mouse by using antigen-specific regulatory T cells derived from recipient mouse.

The effectiveness of these approaches was to be studied by comparing recipient mice of allogeneic and syngeneic bone marrow cells.

Materials and methods

4.1. Materials

4.1.1 Animals

HA mice [B6;129S4-F8tm1Kaz/J], C57BL/6J mice, eGFP expressing C57BL/6J [C57BL/6-Tg(UBCGFP)30Scha/J] mice, FVB/J mice and eGFP expressing FVB/J mice [FVB.Cg-Tg(CAG-EGFP)B5Nagy/J] were used in this study. Among these, HA mice, C57BL/6J mice and UBC-GFP mice expressed $H2K^b$ haplotype of MHC-I, whereas FVB/J mice and FVB-GFP mice expressed $H2K^q$ MHC-I haplotype. Mice were obtained from the Jackson Laboratories and maintained in the institute's experimental animal facility. Mice were kept in an isolator and fed with autoclaved acidified water and irradiated food *ad libitum*. All experiments were conducted as per procedures approved by the Institutional Animal Ethics Committee at the National Institute of Immunology, New Delhi, India.

4.1.2 Reagents and buffers

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

4.1.2.1 PBS (pH7.4)

Dulbecco's PBS (dry powder without Calcium and Magnesium ions, TS1006) was purchased from HiMedia Laboratories, Mumbai, India. The powder was dissolved in MQ water and sterilized by autoclaving and stored at 4°C.

4.1.2.2 Cell culture media

IMDM: IMDM was purchased as dry powder from Gibco (Grand Island, NY, USA) and reconstituted in MQ water. Sodium bicarbonate was added at a concentration of 3.0 g per litre of medium, filter sterilized and stored at 4°C.

DMEM: DMEM was purchased as dry powder from Gibco and reconstituted in MQ water. Sodium bicarbonate was added at a concentration of 3.7 g per litre of medium, filter sterilized and stored at 4°C.

4.1.2.3 MACS buffer

PBS (pH 7.2) containing:

BSA	0.5%

EDTA	2 mM

Filter sterilized, degassed and stored at 4°C.

4.1.2.4 Lineage negative cell isolation kit

The Lineage Cell Depletion Kit (130-090-858, Miltenyi Biotec, Bergisch Gladbach, Germany) is a magnetic labeling system for the depletion of mature hematopoietic cells, such as T cells, B cells, monocytes/macrophages, granulocytes and erythrocytes and their committed precursors from bone marrow. For depletion, cells are magnetically labeled with a cocktail of biotinylated antibodies against a panel of lineage antigens [CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, and Ter-119] and subsequently with Anti-Biotin MicroBeads. A LS MACS column with a maximum labeled cell capacity of 10^8 and maximum total cell capacity of 2×10^9 cells was used to bind the labeled cells.

4.1.2.5 T_{reg} cell isolation kit

$CD4^+CD25^+$ Regulatory T Cell Isolation Kit (130-091-041, Miltenyi Biotec) consists of the following components:

1. $CD4^+CD25^+$ Regulatory T Cell Biotin-Antibody Cocktail which includes biotin-conjugated monoclonal anti-mouse antibodies against:

CD8a	[Ly-2; isotype: rat IgG2a]
CD11b	[Mac-1; isotype: rat IgG2b]
CD45R	[B220; isotype: rat IgG2a]
CD49b	[DX5; isotype: rat IgM]
Ter-119	[isotype: rat IgG2b]

2. Anti-Biotin MicroBeads: MicroBeads conjugated to monoclonal anti-biotin antibody [isotype: mouse IgG1].

3. CD25-PE, mouse: Monoclonal anti-mouse CD25 antibody conjugated to R-Phycoerythrin (PE) [clone: 7D4; isotype: rat IgM].

4.1.2.6 CFSE labeling kit

Vybrant® CFDA SE Cell Tracer kit (V12883, Invitrogen, Carlsbad, CA, USA) consists of:

Component A:	CFDA SE (MW = 557.47)
Component B:	DMSO

4.1.2.6 Reagents for dendritic cell isolation

RPMI-1640:

RPMI-1640 was purchased as dry powder from Gibco and reconstituted in MQ water. Sodium bicarbonate was added at a concentration of 2 g per litre of medium, sterilized by filtration through a 0.22 μm membrane filter (Advanced Microdevices, Ambala, India) and stored at $4^{0}C$.

Complete RPMI-5 medium

RPMI-1640 medium containing:

5% heat-inactivated fetal bovine serum (Biological Industries)

HEPES	10 mM
gentamicin sulfate	20 μg/ml
2-ME	50 μM

Dense BSA:

1. In a 1-liter beaker, 186 ml PBS, 29 ml of 1 N NaOH, and 65 ml water (care was taken to avoid splashing the solution onto the inner walls of the beaker) were taken. Without stirring, layered 106 g BSA (Cohn fraction V) onto the surface of the solution. Covered the beaker with foil and refrigerated overnight, allowing the albumin to dissolve slowly.

2. The next day, gently swirled the clear, honey-brown solution, and filter sterilized the solution first through a 0.4 μm membrane filter and then through 0.22 μm membrane filter using positive pressure. Stored for up to 3 months at $4^{0}C$.

3. The density of BSA will be 1.080 g/ml.

4.1.2.7 Antibodies

Antigen	Catalogue number and manufacturer
Polyclonal rabbit anti-mouse FVIII light chain	(sc-33584; Santa Cruz Biotechnology Inc., CA, USA)
Polyclonal rabbit anti-mouse FVIII heavy chain	(sc-33583; Santa Cruz Biotechnology Inc. CA, USA)

Mouse anti-GFP	(SAB-500, Stressgen, NY, USA)
Mouse anti-cytokeratin-18 (CK-18)	(CK-18; sc-32329, Santa Cruz Biotechnology)
Goat anti- vWF	(sc-8086; Santa Cruz Biotechnology)
Rat anti-GFP	(338001, Biolegend, CA, USA)
Biotin anti-CD31	(102404, Biolegend)
Goat anti-albumin	(A90-234A Bethyl Laboratories, In., TX, USA)
PE-Rat anti-CD3	(555275, BD Pharmingen, CA, USA)

4.1.2.8 Reagents for ELISA

Carbonate bicarbonate coating buffer (0.1 M), pH 9.6:

Na_2CO_3	3.03 g
$NaHCO_3$	6.0 g
MQ water	1000 ml

Blocking buffer

PBS containing 5% horse serum (Biological Industries, Kibbutz Beit HaEmek, Israel) was used to block wells after coating.

Washing buffer

PBS containing 5% horse serum and 0.05% Tween-20 was used for washing the plates.

Substrate

BD OptEIA™ TMB substrate reagent set (Becton, Dickinson and Company, NJ, USA) was used as substrate. Equal volumes of Solution A and Solution B were mixed well immediately before use.

4.1.2.9 Reagents for COATEST

COATEST® SP4 FVIII kit (82 4094 63, Chromogenix Instrumentation Laboratory, Milano, Italy) contained the following components:

1. S-2765 15.4 mg + I-2581 Chromogenic substrate (N-a-Z-D-Arg-Gly-Arg-pNA), 15.4 mg and synthetic thrombin inhibitor, 0.4 mg.
2. Factor IXa + factor X 2.7 IU Lyophilized bovine factors IXa and X.
3. CaCl2 Calcium chloride solution, 0.025 M
4. Buffer, 10X stock solution Concentrated Tris buffer containing NaCl and BSA. 1X buffer will contain Tris 0.05 M, pH 7.3, 10 mg/l Ciprofloxacin and 1.0% BSA.
5. Phospholipid Mixture of highly purified phospholipids and 10 mg/l Ciprofloxacin.

4.1.2.10 Reagents for Immunocytochemistry

PBS-azide:

PBS containing 0.1% sodium azide.

Permeabilization/blocking buffer

PBS containing:

Saponin	0.1%
Ovalbumin	0.5%
Sodium azide	0.1%

Washing buffer

PBS containing:

Saponin	0.05%
Ovalbumin	0.5%
Sodium azide	0.1%

4.1.2.11 Reagents for Immunohistochemistry

PBS-Tween:

PBS containing 0.05% Tween-20.

Peroxidase blocking solution:

Mixed 30% H_2O_2 in PBS to form a 0.3% solution which was used to inactivate endogenous peroxidase activity during immunohistochemical staining of liver sections.

Solutions used for antigen retrieval:

Trypsin Stock Solution (0.5% in Distilled Water):

Trypsin	50 mg
MQ	10 ml

Mixed to dissolve and stored at -20^{0}C.

Calcium Chloride Stock Solution (1%):

Calcium chloride	0.1 g
Distilled water	10 ml

Mixed well and stored at 4^{0}C.

Trypsin Working Solution (0.05%):

Trypsin stock solution (0.5%)	1 ml
Calcium chloride stock solution 1%	1 ml
MQ water	8 ml

Adjusted pH to 7.8 with 1N NaOH. Stored at 4^{0}C for one month or -20^{0}C for longer term.

4.1.2.12 Reagents for the 2-step liver perfusion

Ca^{2+}/Mg^{2+} free perfusion buffer

HEPES	10 mM
KCl	3 mM
NaCl	130 mM
NaH_2PO4/H_2O	1 mM
D-glucose	10 mM
pH	7.4

Stock percoll solution

9 parts of percoll solution was mixed with 1 part of 10X PBS, mixed well and stored at 4^{0}C.

4.1.2.13 RNA gel (denaturing)

10 X MOPS buffer	5 ml
DEPC treated water	37 ml

Added 1 g agarose, mixed well and boiled till all agarose particles dissolved. The solution was cooled to about 60^{0}C, and added 8 ml of 40 % formaldehyde. Added ethidium bromide (0.2 μg/ml), mixed well and poured in to the mould.

10X MOPS buffer:

MOPS	41.8 g
Sterile DEPC-treated H_2O	700 ml

Adjust the pH to 7.0 with 2 N NaOH.

DEPC-treated 1 M sodium acetate	20 ml
DEPC-treated 0.5 M EDTA (pH 8.0)	20 ml

Adjust the volume to 1 liter with DEPC-treated H_2O.

4.1.2.14 DNA gel

PCR products were run in 1.6% to 2.5% agarose gels in TBE buffer.

10X TBE:

Tris Base	108 g
Boric Acid	55 g
EDTA	9.3 g
MQ water	1000 ml

4.1.2.15 TEM

Blocking Buffer (1% BSA, 3% NGS, 0.04% Triton in PBS)

Skimmed milk powder	2 g
PBS	100 ml

Stir to dissolve.

Washing Buffer

To prepare 100 ml,

Fish gelatin	1.0 ml
PBS	100 ml

5% Uranyl Acetate Solution

$UO_2(CH_3COO)_2 \cdot 2H_2O$	2.5 g
MQ water	50 ml

Covered with foil and stirred overnight.

Added 10 drops of glacial acetic acid and Stored the solution at 4°C.

Reynold's Lead Citrate Solution

Added chemicals in distilled water in following order

$Pb(NO_3)_2$	1.33 g
$(Na_3C_6H_5O_7.2H_2O)$	1.76 g
1N NaOH	5 ml
MQ water	30 ml

Stirred for 10 minutes to dissolve and added an additional 15 ml of MQ water. Stored the solution at 4°C.

4.1.3 RT-PCR and Real Time RT-PCR

Primers:

For PCR, primer pairs, except for FVIII mutant and wild type (WT) alleles, were designed using Primer Designing Tool of National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD, USA) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Sigma-Aldrich. FVIII mutant and WT alleles were synthesized as per the sequences given in the genotyping protocol of the Jackson Laboratory (Bar Harbor, ME, USA) by Microsynth AG (Balgach Schweiz, Switzerland).

Primers for Real Time RT-PCR were designed using the Primer Express® software version 2.0 (Applied Biosystems, CA, USA) and synthesized by Sigma-Aldrich.

Gene	Forward primer	Reverse primer	Ta [°C]	Amplicon size [bp]
Albumin	5'-GTGCAAGAACTATGCTGAGG-3'	5'-ACTCACTGGGGTCTTCTCAT-3'	61.5	466 bp
GAPDH	5'-CCTGCACCACCAACTGCTTAG-3'	5'-GGGTGGCAGTGATGGCAT-3'	60	102 bp
FVIII mutant allele	5'-TGTGTCCCGCCCCTTCCTTT-3'	5'-GAGCAAATTCCTGTACTGAC-3'	61.2	420 bp
FVIII WT allele	5'-TGCAAGGCCTGGGCTTATTT-3'	5'-GAGCAAATTCCTGTACTGAC-3'	61.2	620 bp
FVIII A3 domain	5'-GCCTGGGCTTATTTCTCTGATG-3'	5'-TGAGCAGGATTCAGTGTGTTCG-3'	60	101 bp
vWF	5'-ACAGACGCCATCTCCAGATTCA-3'	5'-TGTTCATCAAATGGTGGGCAGC-3'	64	572 bp
β-actin	5'-AGCCATGTACGTAGCCATCC-3'	5'-CTCTCAGCTGTGGTGGTGAA-3'	57.8	228 bp

4.2 Methods

4.2.1 Acute liver injury model

Acute liver injury in HA mice was induced by IP injection of acetaminophen at a dose of 500 mg/kg body weight (Yadav *et al.*, 2009).

1. Six to eight week old HA mice were subjected to acute liver injury.

2. The mice were weighed and 50 mg/ml solution of acetaminophen was injected IP at a dose of 10 μl per g body weight using a syringe and a 26G ½ needle.

4.2.2 Generation of bone marrow chimera

1. Six to eight week old HA mice were irradiated at 960 cGy and transplanted IV with 10 x 10^6 unfractionated bone marrow cells from FVB/J mice (Streetz *et al.*, 2008).

2. Simultaneously, another group of HA mice were similarly irradiated and transplanted IV with an equal number of unfractionated bone marrow cells from FVB-GFP mice.

3. The mice were housed at pathogen-free conditions and fed sterile water and food.

4. The number of mice surviving after a given time is recorded.

5. Peripheral blood mononuclear cells were collected from FVB-GFP bone marrow transplanted mice one month after transplantation, stained for CD3 and analyzed using FACS to ascertain the percentage of GFP^+CD3^+ cells to determine the extent of chimerism.

4.2.3 Dexamethasone mediated immune suppression

1. Six to eight week old HA mice were subjected to immunosuppression by IM injection of 8 mg/kg dexamethasone sodium phosphate (Decdan™, Wockhardt Ltd., Mumbai, India) which was repeated every third day.

2. One day after the first injection, the mice were subjected to liver damage as described elsewhere [section 3.2.1].

3. Twenty four hours after liver damage, the mice were transplanted IV with 0.25 x 10^6 Lin^- BMCs from FVB-GFP mice.

4. The mice were housed in an isolator and maintained on a diet of sterilized water and food.

5. The mice were bled from retroorbital plexus after one week and any change in $CD3^+$ cell count was determined by flow cytometry.

4.2.4 Lineage negative cell isolation and RO transplantation

4.2.4.1 The Lin^- BMCs were isolated using Lineage cell depletion kit- mouse (Miltenyi Biotec) following the manufacturer's protocol.

Preparation of bone marrow cells:

1. BMCs were collected from donor mice femurs and tibias by flushing the shaft with MACS buffer using a syringe and 26G ½ needle.

2. Cells were disaggregated by gentle pipeting for several times.

3. Cells were passed through a 30 µm pre-separation filter (Miltenyi Biotec) to remove cell clumps.

4. Washed the cells, centrifuged at 300 X g for 10 minutes at 4°C. Pipette off the supernatant.

5. Resuspended the cell pellet in IMDM containing 3% FCS and cell number was determined.

Magnetic Labeling:

1. Pelleted the cells by centrifugation at 300 X g for 10 minutes at 4°C. Pipette off the supernatant completely.

2. Resuspended the cell pellet in 40 µl of buffer per 10^7 total cells.

3. Added 10 µl of Biotin-Antibody cocktail per 10^7 total cells.

4. Mixed well and incubated for 10 minutes at 4°C.

5. Added 30 µl of buffer per 10^7 total cells.

6. Added 20 µl of Anti-Biotin MicroBeads per 10^7 total cells.

7. Mixed well and incubated for an additional 15 minutes at 4°C.

8. Washed cells by adding 1-2 ml of buffer per 10^7 cells and centrifuged at 300 X g for 10 minutes. Pipette off supernatant completely.

9. Resuspended the cells in 500 µl of buffer.

Magnetic separation:

1. Placed an LS column in the magnetic field of a MACS separator.

2. Prepared column by rinsing with 3 ml of buffer.

3. Applied the cell suspension on to the column.

4. Allowed the cells to pass through and collected negative fraction in a tube containing IMDM with 10% FCS.

5. Washed the column by adding 3 ml of buffer 3 times.

6. Collected the effluent in the same tube. This represented the enriched lineage negative cell fraction of BM.

4.2.4.2 The Lin^- cells were resuspended in serum-free IMDM and transplanted in to the recipient mice at a dose of 0.25×10^6 cells per mouse by the RO route.

1. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (15 mg/kg).

2. The recipient mouse was laid in lateral recumbency and its head secured.

3. The cell suspension in a volume of 100 µl is injected through retro-orbital sinus using a 1 ml syringe connected to a 27G ½ needle.

4.2.5 Dendritic cell isolation

Dendritic cells from FVB/J mice were isolated as described elsewhere (Inaba *et al.*, 2001).

Preparation of low-density splenocytes:

1. Mechanically disrupted whole spleens between two frosted glass slides.

2. Collected the cells in serum-free RPMI-1640 medium and resuspended by pipetting.

3. Centrifuged splenocyte suspension for 10 minutes at 280 X g at 4^0C and aspirated the supernatant.

4. Promptly resuspended the cell pellet in dense BSA, 1 ml per spleen.

5. Prepared BSA columns by transferring 5 to 6 ml of cell suspension into a 15 ml conical tube and carefully overlaying with 1.5 ml of 4^0C RPMI-1640 medium, forming a sharp interface.

6. Centrifuged the BSA columns for 15 minutes at 9500 × g at 4°C using slow acceleration and keeping the brakes turned off.

7. Carefully collected the cells from the interface region, taking the full volume of RPMI-1640 and the top 1 ml of BSA.

8. Pooled the cells from BSA columns into a single fresh 50 ml conical tube. Filled the 50 ml tube with 4°C RPMI-1640 to dilute the BSA and mixed by gentle inversion.

9. Centrifuged for 10 minutes at 280 × g at 4°C and aspirated the supernatants.

10. Resuspended the cells in 5 to 10 ml complete RPMI-5 medium per interface and placed them on ice.

11. Counted viable cells by trypan blue dye exclusion method.

Enrichment of dendritic cells by plastic adherence

12. Adjusted the cell density to 10^7 cells/ml using complete RPMI-5.

13. Plated 4 ml of this suspension per 60 mm tissue culture dish.

14. Incubated the dishes 90 minutes, allowing the dendritic cells to adhere.

15. Removed and discarded nonadherent cells by gently washing the surface of each dish with 37°C RPMI-1640 until all regions of its surface changed from rough and turbid to smooth and nearly clear.

16. Repeated this wash once. Covered each dish with 4 ml fresh 37°C RPMI-1640 and incubate 30 to 60 minutes to release additional contaminating lymphocytes.

17. Repeated the wash as in step 9.

18. Removed the medium from each washed dish, replaced it with 4 ml fresh complete RPMI-5, and incubated the dishes for 12 to 20 hours to allow the initially adherent dendritic cells to detach.

19. Washed the surface of each dish with 37°C supplemented RPMI-5.

20. Pooled the eluted cells into 15 ml conical tubes on ice.

21. Rinsed each dish with 2 ml complete RPMI-5 and pooled the rinses.

22. Centrifuged the collected cells for 10 minutes at 280 × g at 4°C and discarded the supernatant.

23. Resuspended the cell pellet in 1 ml fresh complete RPMI-5 per tube and placed on ice. Counted viable cells by trypan blue dye exclusion method.

4.2.6 Isolation of T_{reg}s

$CD4^+CD25^+$ T_{reg}s were isolated by MACS from total splenocyte population of HA mice using a 2-step protocol. In the first step, $CD4^+$ cells were isolated by using $CD4^+CD25^+$ Regulatory T Cell Isolation Kit- mouse (Miltenyi Biotec) following the manufacturer's protocol. Along with this, the cells were labelled with CD25-PE antibody. In the second step, the $CD25^+$ cells were isolated from the $CD4^+$ cell population by FACS.

Splenocyte labeling

1. Prepared a single cell suspension from spleen of HA mouse by mechanical disruption of the spleen between the frosted ends of two sterilized glass slides. Determined the number of splenocytes isolated.
2. The cells were passed through a 30 μm nylon mesh.
3. Centrifuged the cells at 300 × g for 10 minutes.
4. The supernatant was aspirated completely and the cell pellet was resuspended in 40 μl of buffer per 10^7 total cells.
5. Added 10 μl of $CD4^-$ Biotin-Antibody Cocktail per 10^7 total cells. Mixed well and refrigerated for 10 minutes at 4°C.
6. Added 30 μl of buffer, 20 μl of Anti-Biotin MicroBeads and 10 μl of CD25-PE antibody per 10^7 total cells. Mixed well and refrigerated for an additional 15 minutes in the dark at 4°C.
7. Washed cells by adding 2 ml of buffer per 10^7 total cells and centrifuged at 300 × g for 10 minutes.
8. Aspirated supernatant completely and resuspended cell pellet in 500 μl buffer.

Immunomagnetic separation:

9. Placed LD Column in the magnetic field of a MACS Separator.
10. Prepared column by rinsing with 2 ml of buffer.
11. Applied cell suspension onto the column.

12. Collected unlabeled cells which pass through and washed column with 2 X 1 ml of buffer. Performed washing steps by adding buffer successively once the column reservoir was empty. Collected total effluent which contained the unlabeled $CD4^+$ T cell fraction.

13. The $CD4^+CD25^{+/-}$ cells were sorted using BD FACSAria III Cell Sorter (Becton, Dickinson and Company, NJ, USA) in to $CD4^+CD25^+$ cells and $CD4^+CD25^-$ cells.

14. The purity of separated fractions was determined by flow cytometry.

15. The viability of cells was determined by trypan blue dye exclusion method.

4.2.7 Assessment of T_{reg} *in vitro* activity

CFSE labeling of T_h cells

Cell labeling was done using Vybrant® CFDA SE cell tracer kit according to the manufacturer's instructions:

1. Centrifuged to obtain a cell pellet and aspirated the supernatant.
2. Resuspended the cells gently in prewarmed (37°C) PBS containing CFSE at a concentration of 5 µM.
3. Incubated the cells for 15 minutes at 37°C.
4. Re-pelleted the cells by centrifugation and resuspended in fresh prewarmed DMEM.
5. Incubated the cells for another 30 minutes to ensure complete modification of the probe and then washed the cells again.

T_{reg} mediated suppression of CFSE dilution

T_{reg} mediated immune suppression was evaluated *in vitro* according to published protocol (Venken *et al.*, 2007) with slight modifications. Briefly:

1. CFSE labeled T_h cells were added to a 96 well plate at the following concentrations: 5×10^5, 2.5×10^5, 1.25×10^5, 6.25×10^4, and 3×10^4 cells per well.
2. For polyclonal activation of CFSE labeled T_h cells, PHA was added at a concentration of 10 µg/ml in serum-free DMEM to each well.
3. A constant number of 2×10^4 T_{reg}s were added in to each well.
4. The resulting ratios of T_h cells to T_{reg}s were: 25:1, 12.5:1, 6.25:1, 3:1 and 1.5:1.
5. The cell mixtures were cultured for 4 days at 37°C at 5% CO_2 tension.

6. The cultures were analyzed for inhibition of CFSE dilution by flow cytometry.

T_{reg} *in vitro* sensitization

1. $CD4^{+}CD25^{+}$ T_{reg}s were freshly isolated from HA mouse spleen.

2. Dendritic cells were isolated from FVB/J mouse spleen.

3. T_{reg}s were cultured for 2 days along with irradiated dendritic cells (1500 cGy) in the presence of 60 ng/ml IL-2.

4. The mixture of cells was aspirated, pelleted at 300 X g for 10 minutes and transplanted along with Lin^{-} BMCs from FVB-GFP mouse in to a liver damaged HA mouse.

4.2.8 Immunofluorescence staining of liver tissue

Preparation of liver tissue blocks:

1. Liver tissue was cut in to approximately 3 mm X 3 mm pieces and fixed in 4% PFA for 5 minutes.

2. Decanted the PFA solution and immersed the tissue pieces in PBS.

3. The tissue pieces were once again immersed in 4% PFA for 5 minutes.

4. Repeated step 2.

5. Decanted and removed the PBS as much as possible.

6. Immersed the tissue pieces in 30% sucrose in PBS solution till the pieces sank (overnight).

7. Removed the pieces, briefly immersed in Jung tissue freezing medium® (Leica Microsystems, Heidelberg, Germany) to remove sucrose.

8. Transferred to a mould and poured tissue freezing medium over the pieces to cover.

9. Frozen the tissues at -20°C to form blocks.

Cryosectioning:

10. Fixed the tissue blocks in a holder fitted to a Shandon Cryotome E (Thermo Electron Corporation, Waltham, MA, USA) and 5 µm sections were made at the optimum cutting temperature.

11. The sections were collected on poly-L-Lysine coated glass slides and preserved at -20°C.

Immunostaining:

12. The sections were thawed, placed in a humidified chamber and covered with PBS for 5 minutes to wash off tissue freezing medium.

13. Pipette out the PBS and added 4% PFA. Incubated the slides for 30 minutes at room temperature.

14. After fixing, removed the PFA, added permeabilization/blocking buffer and incubated at room temperature for 30 minutes to achieve permeabilization of cells.

15. Removed the permeabilization/blocking buffer and added primary antibody diluted to the desired concentration in permeabilization/blocking buffer and incubated for 1 hour at room temperature or overnight at 4^oC.

16. Washed the sections 3 times with washing buffer. Each time, covered the section with washing buffer, kept at room temperature for 5 minutes and aspirated the buffer.

17. Added secondary antibody diluted to the desired concentration in permeabilization/blocking buffer. Incubated for 1 hour at room temperature.

18. Repeated step 16.

19. If there is a tertiary antibody or conjugate, repeated step 17 with the desired concentration of the concerned antibody and then step 18.

20. After the final washing, added DAPI at 10 μg/ml concentration on to the sections and incubated the sections for 5 minutes in the dark.

21. Washed once with PBS.

22. Covered the sections with mounting medium over which coverslips were placed.

23. Examined the sections with an Olympus inverted fluorescence microscope (IX51) and a DP70 digital CCD camera over LCPlanFl 20X and 60X objectives and with a Zeiss LSM 510 META confocal laser-scanning microscope using a Plan- Apochromat 63 X/1.4 oil objective. DP controller and LSM 510 software were used for acquisition of images by the Olympus and Zeiss microscopes, respectively.

24. When cells in suspension were to be analyzed by fluorescence microscopy for intracellular proteins, the cells were coated over a limited area on a poly-L-Lysine coated glass slide by centrifugation in a Cytospin 2 apparatus (Shandon Southern Products, Cheshire,

UK). They were then fixed as described in step 13. They were then permeabilized and staining was continued as described in the rest of the protocol.

4.2.9 Immunocytochemistry and flow cytometry

Immunostaining of intracellular proteins was done according to the following protocol:

1. Cells to be stained were centrifuged at 600 X g for 5 minutes. The supernatant was then decanted and cells were resuspended in PBS-azide.
2. After cell counting, the cells were transferred in to a 96 well round bottom plate at a rate of 1-2 X 10^6 cells per well. Care was taken to ensure that the wells are separated from each other by at least one well.
3. Cells were again spun down and the supernatant discarded.
4. Cells were fixed by resuspending them in 4% PFA and incubating at 4^oC for 30 minutes.
5. After fixing, PFA was removed by centrifugation.
6. Added the permeabilization/blocking buffer, incubated for another 30 minutes at 4^oC and centrifuged the cells at 600 X g for 5 minutes. Removed the supernatant.
7. To the sample wells, added 50 µl of the desired dilution of primary antibody in permeabilization/blocking buffer. To the control wells 50 µl of the buffer was added. Incubated the plate for 45 minutes at 4^oC.
8. Washed the cells by adding 100 µl of washing buffer, mixing and then centrifuged at 600 X g for 5 minutes. Washing was repeated 3 times, each time allowing 5 minutes of incubation after mixing.
9. Added the secondary antibody, diluted in permeabilization/blocking buffer to the desired concentration, 50 µl for each well, to the test wells and secondary antibody control wells.
10. Incubated the plate for 45 minutes at 4^oC.
11. Washed 3 times as described in step 8.
12. Resuspended in PBS-azide and stored at 4^oC till analysis in a flow cytometer.

Immunostaining of cell membrane proteins was done using the above protocol with the following modifications:

1. Fixing step before staining was avoided.

2. Permeabilization was not done. Instead a blocking buffer without detergents was used for blocking for 30 minutes at 4°C.

3. Washing buffer did not contain any detergents.

4. After the staining was completed, the cells were fixed using 4% PFA and stored at 4°C until analysis by flow cytometry.

4.2.10 Immunohistochemical study of recipient mouse liver

Liver tissue blocks were prepared as described earlier (section 4.2.7.1).

1. Five-micrometer serial sections were permeabilized with buffer containing 1% bovine serum albumin and 0.15% Triton X-100 in phosphate-buffered saline (PBS) for 30 minutes at room temperature.

2. The sections were then stained with mouse anti-GFP or rabbit anti-GFP, mouse anti-cytokeratin-18, and goat anti-von Willebrand factor (vWF) antibodies for 1 hour at room temperature.

3. The sections were then incubated with secondary antibodies conjugated to Alexa Fluor 488, 546, or 594 (Molecular Probes, Inc., Eugene, OR, USA) for 1 hour at room temperature.

4. For FVIII staining, tissue sections were blocked in 2% goat serum and stained with rabbit anti-FVIII light chain specific antibody or the corresponding isotype control serum (S-5000; Vector Laboratories, Burlingame, CA, USA). Sections were then incubated with Alexa Fluor 546- conjugated goat anti-rabbit IgG, and the nuclei were stained with DAPI.

5. Stained sections were imaged with an Olympus fluorescence microscope (IX51) and Zeiss LSM 510 META confocal Laser-scanning microscope.

4.2.11 Immunohistochemistry

Paraffin sectioning:

1. Liver tissue was fixed in 10 % buffered formalin for 48 hours.

2. Tissue was washed with running tap water for 15 minutes.

3. Tissue was dehydrated in different grades of isopropanol:

a. 50% 30 minutes.

b. 70% 30 minutes.

c. 90% 30 minutes.

d. 100% 1 hour.

4. Tissue was then immersed in a mixture of xylene and isopropanol (1:1) for 30 minutes. This was followed with immersion in:

a. Xylene 1 hour.

b. Repeated step 4a.

c. Paraffin wax at 60°C. 1 hour.

d. Repeated step 4c twice.

5. The tissue was embedded in paraffin wax in a suitable mould.

6. Removed the mould, mounted the block on a microtome (MRS3500, Histo-Line Laboratories, Milan, Italy) and cut 5 μm sections.

Deparaffinization:

Immersed the slide in the following reagents successively:

7. Xylene 5 minutes.

8. 100% isopropanol 5 minutes.

9. 90% isopropanol 5 minutes.

10. 70% isopropanol 5 minutes.

11. Slides were rinsed in MQ.

Antigen retrieval and immunostaining:

12. Covered the sections with trypsin (27250-018, Gibco, Grand Island, NY, USA) working solution and incubate for 10-20 minutes at 37°C.

13. Allowed the sections to cool at room temperature for 10 minutes.

14. Rinsed the sections in PBS Tween for 2 minutes.

15. Repeated step 14.

16. Blocked the sections with 2% goat serum for 30 minutes.

17. Incubated the sections with primary antibody diluted with PBS Tween to the desired concentration for 1 hour at room temperature.

18. Rinsed sections with PBS Tween for 2 minutes.

19. Repeated step 18.

20. Blocked the sections with peroxidase blocking solution for 8 minutes.

21. Rinsed with PBS Tween for 2 minutes.

22. Repeated step 21 twice.

23. Incubated the sections with secondary antibody diluted with PBS Tween to the desired concentration for 1 hour at room temperature.

24. Washed the sections in PBS 3 times for 2 minutes each.

25. Incubated the sections with DAB substrate solution (S-4100, Vector Laboratories, Burlingame, CA, USA) for 10 minutes at room temperature.

26. Washed the sections with MQ water for 5 minutes.

27. Incubated the sections in Mayer's hematoxylin solution for 5 minutes at room temperature.

28. Washed the cells with MQ for 5 minutes.

29. Immersed the slides in 90% isopropanol for 2 minutes.

30. Immersed the slides in 100% isopropanol for 5 minutes.

31. Transferred the slides to xylene for 5-10 minutes.

32. DPX mountant was placed over the sections and coverslips were mounted.

4.2.12 ELISA

ELISA was performed on the basis of a published protocol (Follenzi *et al.*, 2008), with minor modifications:

1. A 96-well microtiter plate was coated with 100 µl of diluted mouse plasma (1:80) in carbonate-bicarbonate buffer by incubating at 4^{0}C overnight.

2. The plate was washed with washing buffer.

3. Added 200 µl of blocking buffer in to each well and incubated the plate at 37^{0}C for 1 hour.

4. Removed the blocking buffer. Washed the plate three times with washing buffer.

5. Added 100 µl of rabbit anti-mouse FVIII polyclonal antibody at 1:3000 dilution.

6. Incubated the plate at 37^{0}C for 2 hours.

7. Discarded the antibody solution and washed the plate three times with washing buffer.

8. Added 100 µl goat anti-rabbit IgG-HRP (1:1000) and incubated the plate at 37^{0}C for 1 hour.

9. Washed the plate three times with washing buffer.

10. Added 100 µl of TMB substrate was added in each well.

11. Incubated the plate at 37^{0}C in the dark for 15 minutes.

12. Added 50 µl of 2N H_2SO_4 to inactivate the peroxidase, and the plate was read at 450 nm on a PowerWave XS (BioTek Instuments Inc., Winooski, VT, USA) reader.

4.2.13 *In vitro* FVIII activity in mouse plasma

Measurement of FVIII activity in mouse plasma was done using COATEST® SP4 FVIII kit following the manufacturer's protocol with some modifications. Nine parts of freshly drawn venous blood were collected into one part trisodium citrate (3.2%). The samples were centrifuged at 2000 X g for 10-20 minutes at 20^{0}C. The plasma was aspirated and stored at -80 °C.

Pooled WT mouse plasma was used as standard. The experiment was conducted as follows:

1. Prepared the predilutions of standard plasma, test plasma and HA plasma immediately before starting the experiment by adding 10 µl of neat plasma in to a microfuge tube containing 90 µl of 1X buffer [Tris 0.05 M, pH 7.3, 10 mg/l Ciprofloxacin and 1.0% BSA] and kept the dilutions at 4^{0}C.

2. Added 50 µl of Phospholipid + FIXa + FX mixture kept at 4^{0}C to a 96 well- flat bottom microtitre plate to make duplicate wells for all the test plasma samples, standard plasma and HA plasma. Added 50 µl of 1X buffer to two wells marked 'reagent blank'.

3. Added 25 µl of corresponding prediluted sample in to each well, mixed well using a micropipette and incubated at 37^{0}C for 4 to 5 minutes.

4. Added 25 µl of $CaCl_2$ (0.025 M) prewarmed to 37^{0}C, to each well, mixed well and incubated at 37^{0}C for exactly 5 minutes.

5. Added 50 µl of substrate solution, prewarmed to 37^{0}C to each well, mixed well and incubated at 37^{0}C for exactly 5 minutes.

6. Added 25 µl of 20% acetic acid to each well to stop the reaction.

7. Read the absorbance of the samples and standards against the reagent blank at 405 nm and 490 nm in a PowerWave XS spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). Subtracted A_{490} from A_{405} to correct for difference due to the microplate.

Percentages of FVIII activity in test samples normalized for HA samples were calculated using the following formula:

% FVIII activity = 100 x (A_{405} of test plasma-A_{405} of HA plasma) ÷ A_{405} of standard plasma

4.2.14 Whole blood clotting time

Whole blood clotting time was determined by the following protocol (Voigt, 2000):

1. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (15 mg/kg).
2. The recipient mouse was laid in lateral recumbency and its head secured.
3. Blood was drawn in a capillary tube from the retro-orbital plexus of each mouse and the time noted.
4. One end of the loaded capillary tube was gently broken at regular intervals and slowly pulled apart to detect blood clots which appeared as threads connecting the two broken ends. The time taken to form a visible clot was recorded.

4.2.15 Tail cut challenge

Phenotypic correction of hemophilia A was assessed by performing the tail cut challenge experiment (Connelly *et al.*, 1998) as follows:

1. The mice were anaesthetized using a combination of xylazine (15 mg/kg) and ketamine (100 mg/kg).
2. The tips of tails were cut using a sharp surgical knife 1.5 cm from the end and the mice were allowed to bleed freely.
3. The mice were observed for a period of 24 hours. Any death was noted and percentage of survival calculated.

4.2.16 Isolation of hepatocytes and LSECs

To further understand the problem, hepatocyte and LSEC populations were isolated from recipient mice livers and relative quantification of FVIII was carried out in the two populations.

Single cells of liver were obtained by a 2-step collagenase perfusion method (Benten *et al.*, 2005; Follenzi *et al.*, 2008)

1. Mice were anesthetized by intraperitoneal injection of xylazine (15 mg/kg) and ketamine (100 mg/kg).

2. In the first step of perfusion, the liver was perfused with HEPES buffer (50 ml) at 37^{0}C containing 1.9 mg/ml EGTA for 10 to 15 minutes.

3. In the second step, the liver was perfused with 50 ml of buffer containing 0.03% collagenase IV (Sigma-Aldrich) and 5 mM $CaCl_2{\cdot}2H_2O$.

4. The liver capsule was incised and a single-cell suspension was prepared in RPMI-1640 medium by light mechanical disruption.

5. To isolate the hepatocyte fraction, the cells were centrifuged at 50 X g for 5 minutes followed by 2 washings with RPMI-1640.

6. To obtain the nonhepatic cell fraction, the supernatant of first centrifugation was spun down at 350 X g for 10 minutes at 16^{0}C, washed with phosphate-buffered saline (pH 7.4), and finally resuspended in the same buffer.

7. To isolate the liver sinusoidal endothelial cell (LSEC) fraction, nonhepatocyte cells were fractionated using Percoll (Braet *et al.*, 1994; Benten *et al.*, 2005)

a. A Percoll gradient was prepared in a 15 ml tube with 2 ml of 75% stock Percoll solution (diluted with PBS) at the bottom and 1.5 ml of 35% stock Percoll solution at the top.

b. The cells were resuspended in PBS and layered on Percoll for centrifugation at 900 X g for 20 minutes without brakes. The cells separated into 2 bands.

c. The lower layer containing LSECs was carefully aspirated and resuspended in an equal volume of PBS, and the cells were centrifuged at 900 X g for 7 minutes.

8. The viable counts of hepatocytes and endothelial cells were determined using trypan blue dye exclusion method.

4.2.17 RNA isolation

Total RNA was isolated from liver tissue or cells using TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA).

1. For isolation from liver tissue, samples were collected immediately after death, snap frozen in liquid nitrogen and stored at -80°C. The samples were pulverized using pre-cooled mortar and pestle, suspended in TRIZOL® Reagent by vigorous vortexing and stored at -80°C.

2. For isolation from cells, the cells were pelleted at 600 X g for 5 minutes at 4°C, removed the supernatant thoroughly and resuspended vigorously in TRIZOL® Reagent and stored at -80°C.

3. The frozen samples were thawed and incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes.

4. Choloroform (0.2 ml of per ml of TRIZOL® Reagent) was added to each tube, and the tubes were capped tightly and shaken vigorously for 15 seconds.

5. The samples were incubated for 2 to 3 minutes at room temperature and then centrifuged at 12,000 X g for 15 minutes at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, and interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube.

6. RNA was precipitated from the aqueous phase by addition of 0.5 ml of isopropyl alcohol per ml of TRIZOL® Reagent, incubation at room temperature for 10 min and centrifugation at 12000 X g for 10 minutes at 4°C.

7. The supernatant was removed and the RNA pellet was washed once by addition of 1 ml 75% ethanol per ml of TRIZOL® Reagent, followed by vigorous mixing by vortexing and centrifugation at 7500 X g for 5 minutes at 4°C.

8. The RNA pellet was briefly dried and dissolved in RNase free water and stored at -80°C.

9. Up to 5 µg of RNA was run in an agarose gel under denaturing conditions to check the integrity of the RNA sample.

4.2.18 Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription was done using the ProtoScript First Strand cDNA Synthesis Kit (NEB, Beverly, MA, USA) according to the manufacturer's protocol.

1. A cDNA synthesis reaction is set up as described below:

Component	Quantity
RNA	Up to 2 μg
10X RT buffer	2 μl
dNTPs	0.5 mM each
Oligo dT primer	5 μM
RNase inhibitor	10 units
M-MuLV reverse transcriptase	5 units
NFW	To make up to 20 μl

2. Reaction conditions were as below:

a. In a reaction tube, added RNA, oligo dT, dNTP mix, RNase inhibitor and water to make up to 17 μl.

b. Briefly spun and incubated at 70°C for 10 minutes.

c. Spun down and added RT buffer and reverse transcriptase.

d. Mixed well, spun down and incubated at 42°C for 1 hour.

e. Terminated the reaction by heating at 95°C for 5 minutes.

f. Spun down, diluted to 50 μl using nuclease free water and stored at -20°C.

3. Genes of interest were amplified by PCR using the cDNA template and primers selective for target gene segments in a MyCycler thermal cycler (BIO-RAD Laboratories, Hercules, CA, USA). The reaction components were generally as given below:

Component	Quantity
10 X standard Taq Buffer (NEB)	2.0 μl
dNTPs	200 μM
Forward primer	1 μM
Reverse primer	1 μM
Taq DNA polymerase (NEB)	1 IU
NFW	To make up to 20 μl.

4. The amplified products were resolved in a 2.0% - 2.5% agarose gel and visualized by ethidium bromide staining, and images were recorded using a UVP Bio-imaging System (UVP, Upland, CA, USA).

4.2.19 Real-Time RT-PCR

Real-time RT-PCR was performedin 0.2 ml clear flat top PCR® strip caps (Axygen Scientific, CA, USA) in the Mastercycler ep *realplex*^{4} (Eppendorf AG, Hamburg, Germany). Reactions were prepared as given below and carried out in quadruplicates of each 10 µl reaction volume.

Component	Quantity
Power SYBR® Green PCR Master Mix	5 µl
Template cDNA	2 µl
Forward primer	50 µM
Reverse primer	50 µM
MQ	To make up to 10 µl

The program was set as below:

1. 95^{0}C for 10 minutes
2. 95^{0}C for 15 seconds

   60^{0}C for 1 minute

   X 40.
3. 95^{0}C for 15 seconds

   60^{0}C for 15 seconds

   60^{0}C to 95^{0}C 20 minutes (melting ramp)

   95^{0}C 15 seconds

   END

For normalization, the expression level of the housekeeping gene (GAPDH) was measured as an endogenous control. Relative quantification was done using Realplex 2.2 software (Eppendorf) and was expressed as log fold difference.

4.2.20 Transmission electron microscopy

To determine the relative contribution of hepatocytes and LSECs to FVIII production in the transplant recipient mice, a qualitative estimation were done first using transmission electron microscopy. Immunogold labeling of tissue sections was performed according to methods published in the literature (Zacharski and Rosenstein, 1978). All reagents were procured from TAAB Laboratories Equipment Ltd.

1. Ultrathin (70 nm) tissue sections were taken on nickel grids.

2. The sections were initially blocked with 2% skimmed milk and then incubated separately on droplets of a 1:50 dilution anti-albumin or anti-vWF antibodies for 4–6 hours at 6^{0}C to 8^{0}C.

3. After washing the sections with PBS containing 1% fish gelatin (6 X 2 minutes), further incubated the sections for 2 hours with anti-goat IgG conjugated to 10 nm colloidal gold particles.

4. The sections were thoroughly rinsed with PBS containing 1% fish gelatin (6 X 2 minutes).

5. In the second step of immunolabeling, all sections were incubated on droplets of anti-FVIII antibody (1:50) for 4 hours at 6^{0}C to 8^{0}C.

6. The sections were washed and incubated with anti-rabbit IgG conjugated to 40 nm colloidal gold particles (1:20) for 2 hours.

7. The sections were washed and stained with uranyl acetate and lead citrate before microscopic analysis (Morgagni 268D; FEI, OR, USA).

Results and Discussion

Study of engraftment potential of bone marrow-derived cells in syngeneic and allogeneic recipient liver

5.1 Introduction

Hepatocytes, the parenchymal cells of liver, carry out numerous vital functions like production of bile, metabolism of dietary compounds, detoxification, and regulation of glucose levels through glycogen storage. In addition to this, they synthesize and secrete molecules essential for normal functioning of the body (Gebhardt, 1992). Physical or chemical injury to liver resulting in a loss of cell mass triggers a regenerative response contributed to by hepatocytes themselves, liver stem cells and migrating bone marrow stem cells (Sell, 2001; Fausto *et al.*, 2006; Oertel and Shafritz, 2008; Thorgeirsson and Grisham, 2006). Restoration of functional liver mass, when it is not possible by replication of hepatocytes in the affected organ, has been done by transplantation of whole liver, viable hepatocytes or stem cells of various origins (Kung and Forbes, 2009; Sancho-Bru *et al.*, 2009; Gilchrist and Plevris, 2010), the latter option being possible due to the transdifferentiation potential of stem cells. Metabolic and genetic disorders of liver can be effectively cured by introduction of functional hepatocytes from a genetically normal individual. Another option is introduction of BM-derived stem cells from a normal individual and simultaneous induction of a regenerative response in the recipient liver. Yadav *et al.* (2009) demonstrated that phenotypic correction of hemophilia A was possible in a FVIII deficient mouse, by inducing uncommitted BMCs from WT mouse to differentiate in to functional hepatocytes. However, the feasibility of this procedure in an allogeneic transplantation setting was not investigated. Since the majority of human clinical transplantations depend on allogeneic donors, it would be important to carry out this evaluation, and in order to achieve that, the following study was undertaken.

5.2 Methods

Expression of FVIII in WT mouse liver, bone marrow, kidney and spleen was analysed by RT-PCR. Acute liver injury was induced in 6-8 weeks old HA mice and Lin$^-$ BMCs isolated from B6-GFP mice were transplanted. Another batch of HA mice liver-damaged in the same manner was transplanted with Lin$^-$ BMCs isolated from FVB-GFP mice. Three months and 6 months after transplantation, the mice were subjected to extensive analysis for phenotypic changes induced by the transplantation of WT mouse stem cells. The mice were bled and plasma was

collected and stored frozen. The plasma samples were subjected to analysis by ELISA for detection and quantification of FVIII protein. Further, the *in vitro* FVIII activity in the plasma samples was determined using COATEST®. To detect efficiency of blood clotting, blood coagulation time of syngeneic recipient (HAT) mice was determined by capillary coagulation method. To determine the survival after trauma, tail clip challenge was conducted. The mice were sacrificed and presence of GFP expressing hepatocytes in liver was investigated by immunofluorescence of cryosections. Presence of FVIII expressing LSECs was investigated by staining cryosections of liver for vWF and FVIII. Presence of FVIII expressing hepatocytes was studied by immunohistochemistry of liver sections using DAB substrate. Single cell preparations of hepatocytes and LSEC were made from HAT mice livers and examined by TEM for the presence of donor derived cells. Single cell suspensions of both cell types were also subjected to FVIII expression analysis by Real-Time PCR-PCR.

5.3 Results

The HA mice carry a mutated FVIII gene, which is produced by insertion of a neo cassette in to the light chain coding region. The neo sequences replace parts of exon 16 and intron 16. For genotyping of HA mice a combination of the two different sense primers and one common anti-sense primer is used. The sense primers are derived from either an intact exon 16 or the *neo* sequences, and the anti-sense primer is derived from exon 17 (Bi *et al.*, 1996). HA mouse will yield a unique amplicon of 420 bp molecular weight whereas WT mouse will yield an amplicon of 620 bp molecular weight.

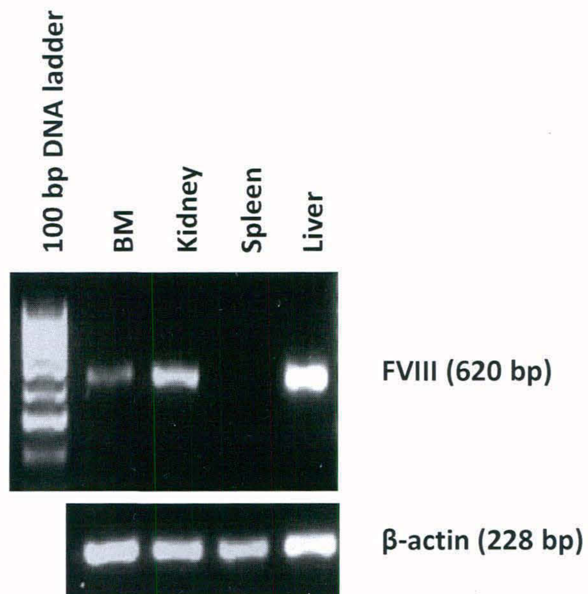
FVIII knockout mice (HA) do not express a functional FVIII molecule. This is because of the truncated light chain of the molecule which resulting from disruption of exon 16 of the gene induced by insertion of the neo cassette. As a result of the disruption alternate splicing generates a truncated mRNA which lack parts of exon 16 and containing the neo cassette sequences and 17 bp of intron 16. No RT-PCR amplification of exon 14 sequences is seen in these mice since reverse transcription across neo sequences is very inefficient (Bi *et al.*, 1996). Hence a primer pair specific for exon 14 sequences (pertaining to the A3 domain of FVIII molecule) could be used for detection of WT mouse bone marrow-derived cells in HA mice. FVIII is expressed primarily in liver by the LSECs and hepatocytes. To appreciate this difference, PCR amplification of FVIII gene from genomic DNA of liver, kidney, spleen and BM

of the WT (donor) mouse was done. Agarose gel electrophoresis of the PCR products showed the presence of the specific amplicon in three of the four tissues analysed (Figure 5.1). These were liver, kidney and BM. However, the expression of FVIII appeared minimal in BM whereas it was highest in liver. No ampification of FVIII gene was found in spleen tissue sample.

Amplificaiton of total liver cDNA was carried out in order to genotypically characterize colonies of HA mice as well as the WT mice used in the following experiments. Amplification of the HA specific sequence including the *neo* sequences was obtained in HA mice. There was no amplification of the WT target sequence in the HA mice. In WT mice there was amplification of WT target sequence and no amplification of HA specific target sequence (Figure 5.2).

In order to ascertain the efficiency of donor cell engraftment in syngeneic transplantation setting, B6-GFP- derived Lin^- BMCs were transplanted in to HA mice ($H2K^b$) (Group I) by intravenous injection after inducing liver damage by intraperitoneal injection of acetaminophen (Yadav *et al.*, 2009). To ascertain the efficiency of donor cell engraftment in allogeneic transplantation setting, FVB-GFP ($H2K^q$) derived Lin^- BMCs were transplantated in to HA mice (Group II) following the same protocol. Control groups of WT and HA mice were liver damaged, but no cells were transplanted in to them. Three months after transplantation, 93% of the Group I mice were alive and healthy (13 out of 14 mice). These mice were sacrificed for analysis of livers by immunofluorescent staining of GFP. Among Group II, only 33% survival was found (2 out of 6 mice). Both of these mice were sacrificed for analysis of livers by immunofluorescent staining. The control HA mice group showed no mortality. WT mice also were observed to show 100% survival over the 3 months period. At the end of 6 months after transplantation, it was found that 80% of the Group I mice survived. Some of the surviving mice were sacrificed and livers were analyzed for the detection of donor derived (GFP-expressing) hepatocytes. Mice in both control groups showed 100% survival (Figure 5.3). Immunofluorescent staining of GFP and albumin revealed presence of donor derived hepatocytes in Group I mice livers, whereas no GFP-expressing hepatocytes were found in Group II mice livers (Figures 5.4 and 5.5).

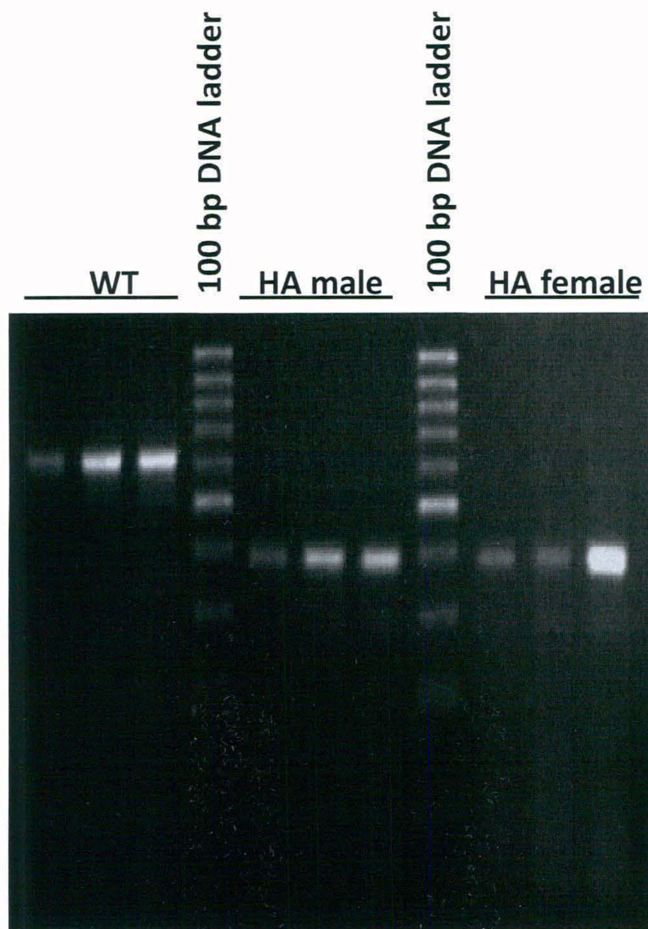
Figure 5.1



Tissue distribution of FVIII

PCR amplification of *FVIII* gene from various tissues of wild type mouse was done. β-actin was used as positive control. (BM) bone marrow.

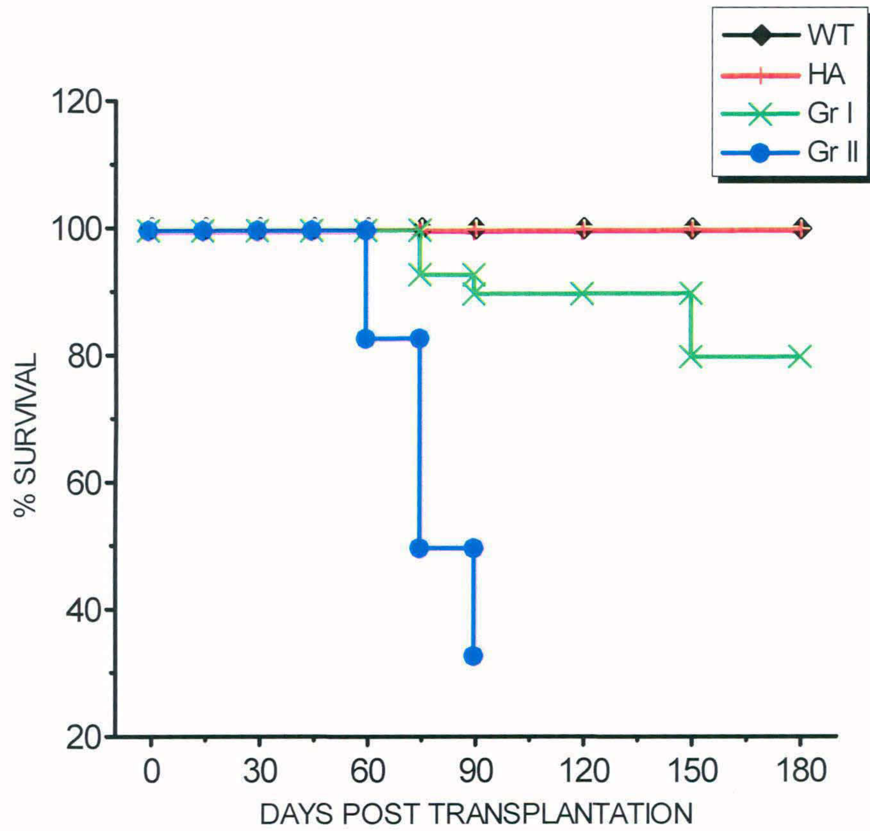
Figure 5.2



Genotyping of wild type and HA mice.

PCR amplification of *FVIII* gene was done from livers of wild type, HA male and HA female mice. (WT) FVB/J . (HA) hemophilia A model.

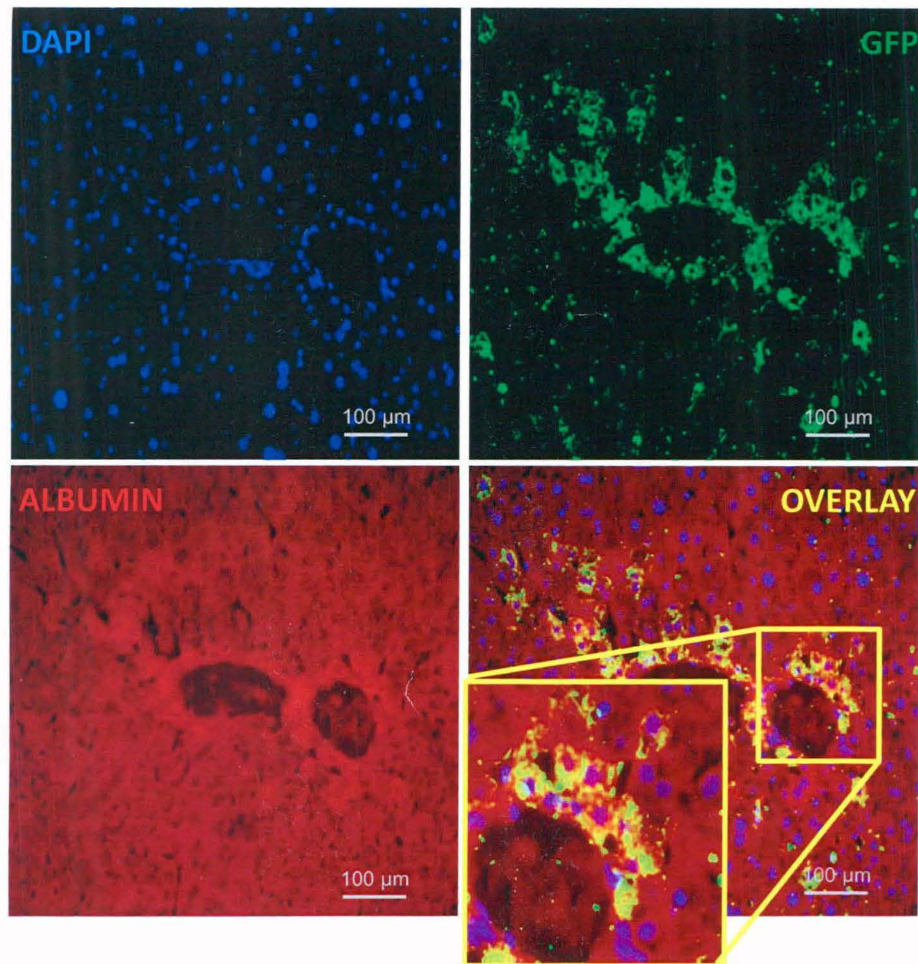
Figure 5.3



Survival graph of Group I and Group II mice.

HA mice were transplanted with syngeneic (Gr I) or allogeneic (Gr II) uncommitted BMCs. The survival following transplantation was recorded

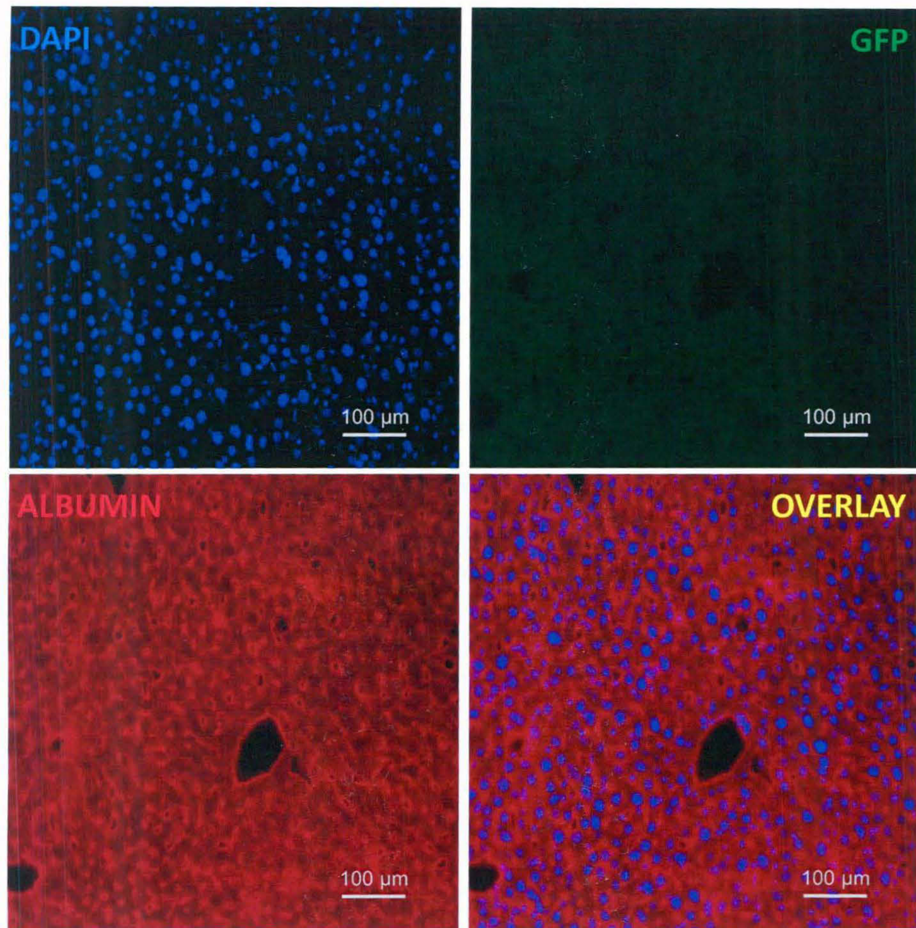
Figure 5.4



Co-expression of GFP and albumin in HAT mouse liver.

HA mice were transplanted with syngeneic uncommitted BMCs. Liver sections were stained for GFP (Green) and albumin (Red) for the presence of donor derived hepatocytes. (HAT) syngeneic transplant recipient. Magnification: 20X

Figure 5.5



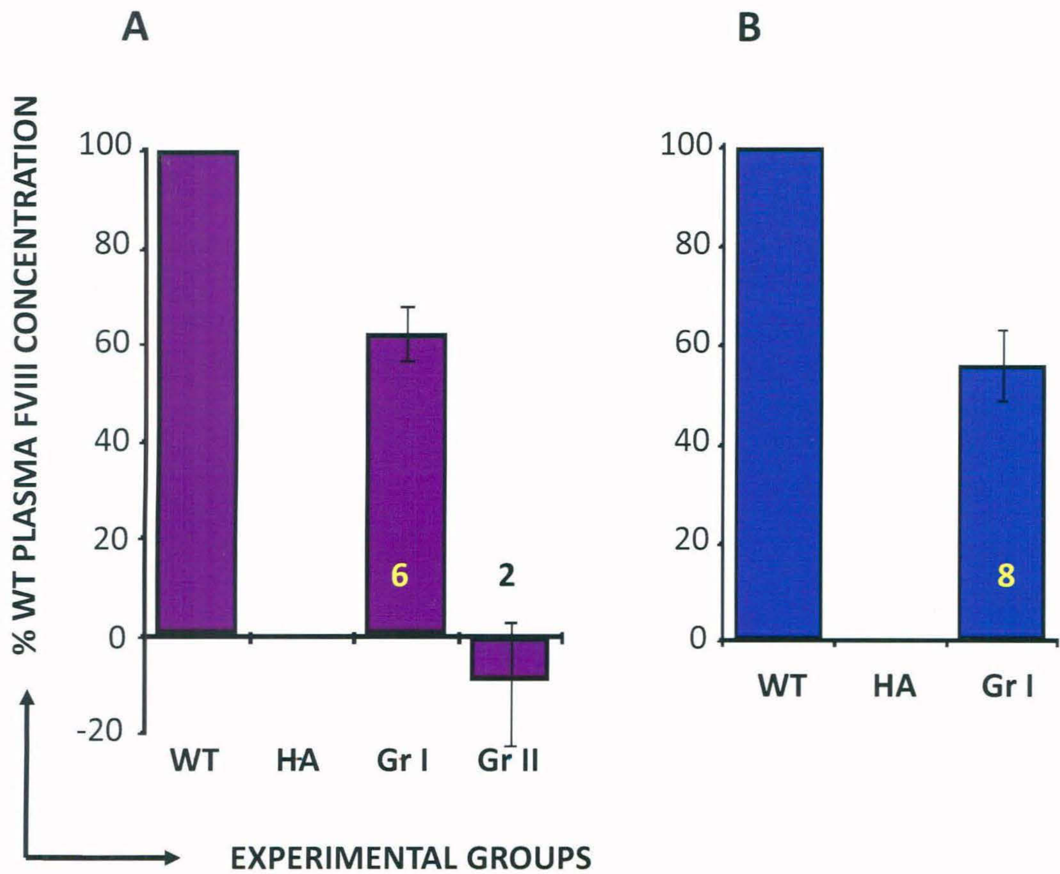
Absence of donor derived hepatocytes in HAT mouse liver after allotransplantation.

HA mice were transplanted with allogeneic uncommitted BMCs. Liver sections were stained for GFP (Green) and albumin (Red) for the presence of donor derived hepatocytes. Magnification: 20X

Mice in Group I and Group II were bled and plasma was collected as described in section 4.2.12 at both 3 month and 6 month time points. The plasma samples were analyzed for the presence of FVIII protein using ELISA. For normalization, the absorbance of pooled HA mouse plasma sample reactions was subtracted from the absorbance of all test and WT mouse control samples. The normalized absorbance of pooled WT mouse plasma sample reactions was taken as 100% and the percentages of plasma FVIII expression of HAT mice samples were calculated as % WT plasma FVIII concentration. The Group I mice, in which syngeneic transplantation was done, showed an increase in concentration to 62.6 ± 5.9% (N=6). In Group II, in which allogeneic transplantation was done, a value of less than 0% with a high standard error was obtained (-9.4 ± 12.63) (N=2). This was considered to be because of the small number of surviving mice in Group II (Figure 5.6A). The increase in plasma FVIII concentration in Group I was significantly more than that in Group II (p <0.005). At 6 months post-transplantation time point, the plasma FVIII concentration in Group I mice was found to have marginally dropped to 55.8 ± 7.3% (Figure 5.6B). This reduction was not considered significant (p>0.05). These results indicated that in the syngeneic recipient, the donor derived liver cells secreted full length FVIII molecule in to blood stream and that FVIII synthesis in recipient liver was stable.

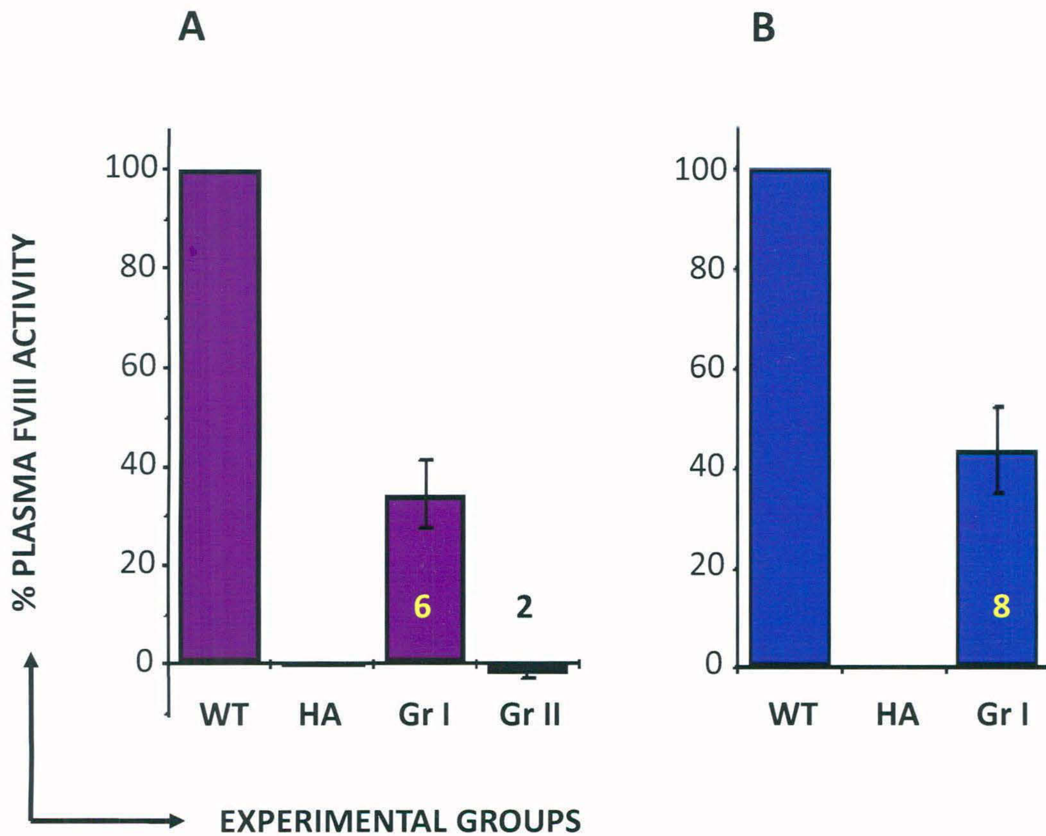
In the intrinsic coagulation pathway, FVIIIa acts as a cofactor for FIXa to convert FX to FXa which converts prothrombin to thrombin (Fay, 2004). In the test conditions, rate of generation of FXa is solely dependent on the amount of FVIII in the test plasma. The FXa hydrolyses a chromogenic substrate to liberate a chromophoric group, and by measuring the intensity of colour, the amount of FVIII present in the test sample can be calculated. To ascertain the coagulation activity of secreted FVIII in HAT mice, COATEST was done and *in vitro* FVIII activity in the test plasma was calculated as described in section 4.2.12. At three months post-transplantation, in Group I, an increase in activity corresponding to 35 ± 7% of WT plasma was observed (N=6). In Group II there was no increase in plasma FVIII activity (-2.3 ± 0.5%, N=2) (Figure 5.7). The augmentation in *in vitro* FVIII activity was significantly more in Group I mice when compared to Group II mice (p <0.05). Six months after transplantation, the *in vitro* plasma FVIII activity was found to have increased marginally to 43.5 ± 8.74. These results suggested that the donor derived hepatocytes secrete a functional FVIII protein in a consistent manner over a long time.

Figure 5.6



Rise in FVIII protein in HAT mouse plasma following transplantation.
Plasma collected from HAT mice was analyzed for FVIII protein concentration using ELISA. WT mouse plasma FVIII concentration was taken as 100% and values for HAT mice were expressed as % wild type plasma FVIII concentration. (A) 3 months post-transplantation. (B) 6 months post transplantation. Number of mouse in each group was mentioned in the respective bars.

Figure 5.7



Rise in FVIII activity in HAT mouse plasma following transplantation.

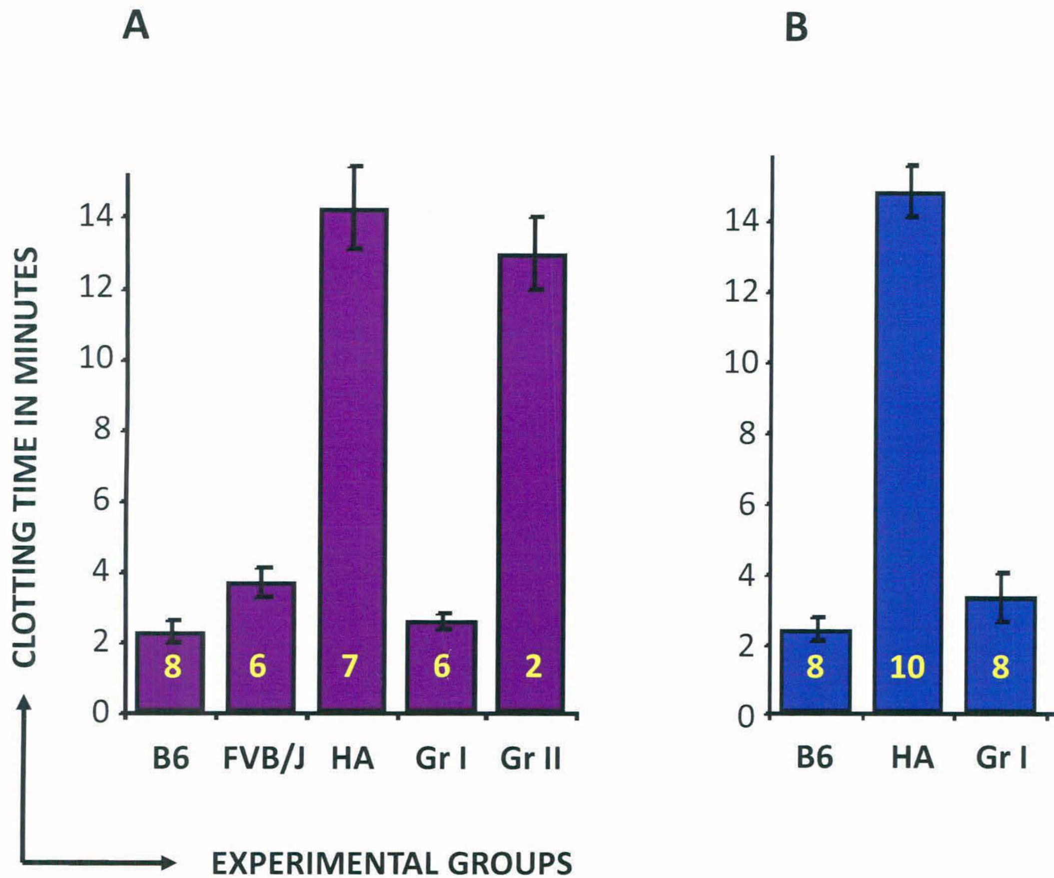
HAT mice plasma was subjected to COATEST. WT mouse plasma FVIII activity was taken as 100% and values for HAT mice were expressed as % wild type plasma FVIII activity. (A) 3 months post-transplantation. (B) 6 months post transplantation. (HA) FVIII^{-}/$^{-}$. (WT) FVB/J. (Gr I) Syngeneic recipient. (GrII) Allogeneic recipient. Number of mouse in each group was mentioned in the respective bars.

In order to ascertain the effect of increased plasma FVIII concentration on the process of blood coagulation in FVIII deficient mice, the experimental groups were subjected to capillary coagulation test which determines the time taken for venous blood to coagulate *ex vivo*. One group each of C57BL/6J mice and FVB/J mice served as positive controls while one group of HA mice was used as a negative control. The tests were conducted as described in section 4.2.13. The results are summarized below in Tables 5.1 and 5.2. Three months post-transplantation, the clotting time of whole blood in Group I mice closely corresponded to that of the MHC matched WT mice (C57BL/6J) (Figure 5.8A). The clotting time in Group II mice was significantly higher when compared to that of the MHC mismatched WT mice (FVB/J). The mice in Group I exhibited a significantly shorter blood clotting time when compared to the mice in Group II ($p<0.005$). Six months post-transplantation, capillary clotting time analysis revealed a minor decrease in *ex vivo* FVIII activity (Figure 5.8B) although this was not significantly different from that of the WT mice ($p>0.05$) or of the earlier time point ($p>0.05$).

Table 5.1: comparison of blood coagulation times of test and control groups 3 months post-transplantation.

Test group	Number of mice	Coagulation time (in minutes)
C57BL/6J [$H2K^b$]	8	2.3 ± 0.4
FVB/J [$H2K^q$]	6	3.7 ± 0.5
HA [$H2K^b$]	7	14.3 ± 1.2
Group I	6	2.6 ± 0.2
Group II	2	13 ± 1

Figure 5.8



Effect of increased plasma FVIII activity in ex vivo blood coagulation time.
HAT mice were subjected to capillary clotting time test. Clotting time in minutes was compared with that of HA and WT mice. (A) 3 months post-transplantation. (B) 6 months post transplantation. (HA) FVIII^{-}/$^{-}$. (Gr I) Syngeneic recipient. (GrII) Allogeneic recipient. Number of mouse in each group was mentioned in the respective bars.

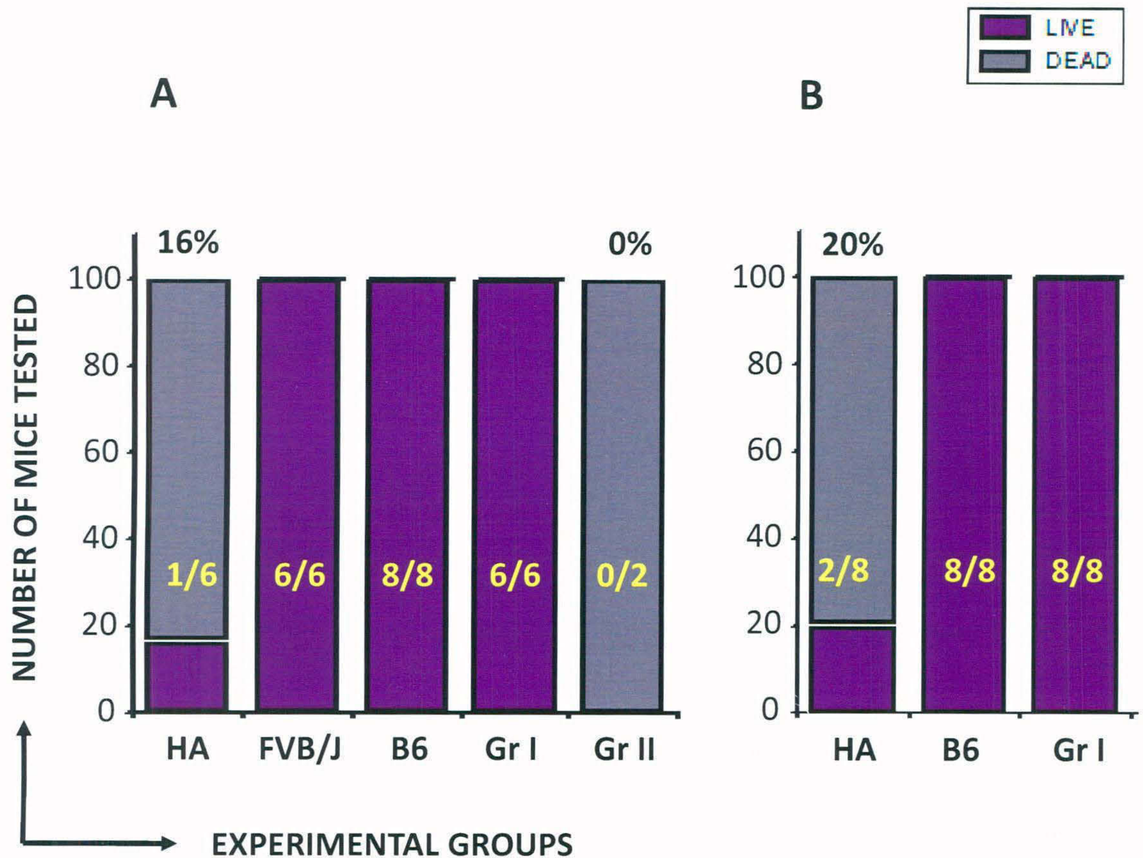
Table 5.2: comparison of blood coagulation times of test and control groups 6 months post-transplantation.

Test group	Number of mice	Coagulation time (in minutes)
C57BL/6J [H2K^b]	9	2.41625 ± 0.33821
HA [H2K^b]	9	14.85556 ± 0.72152
Group I	8	3.34413 ± 0.67392

Further, to analyze the effectiveness of increase in FVIII activity in whole blood in preventing fatal hemorrhage in HAT mice, the two groups of experimental mice were subjected to tail cut challenge as described in section 4.2.14. As controls, one group each of C57BL/6J mice, FVB/J mice and HA mice were also subjected to the test. The results of the tests are summarized in Table 5.3. The WT control groups showed 100% survival whereas all except one mouse died in the HA group resulting in 16%. All the mice in Group I survived the bleeding test whereas the remaining two mice of Group II died of fatal hemorrhage within 24 hours (Figure 5.9). These results indicated that donor derived hepatocytes secrete active FVIII protein in a consistent manner so as to rescue the mice from hemophilia-A phenotype.

FVIII is expressed primarily in liver. The site of biosynthesis of FVIII has been variously reported as hepatocytes and LSECs (Zelechowska *et al.*, 1985). It was assumed that the presence of FVIII in donor cells would indicate the cellular origin of the protein. Since the light chain of FVIII is incompletely expressed in HA mouse, it was expected that immunological detection of the missing part of light chain peptide could reveal the donor derived hepatocytes in the HAT mouse liver. Immunohistochemical analysis of serial sections of formaldehyde-fixed, paraffin-embedded HAT mouse liver tissue was done as described in

Figure 5.9



Effect of increased plasma FVIII activity in phenotypic correction of hemophilia A. HAT mice were subjected to tail cut challenge. The mice remaining alive after 24 hours were considered to have undergone phenotypic correction. Figures inside columns indicate number of mice survived out of the total test mice. Survival percentages other than 100% are given above the columns. (A) 3 months post-transplantation. (B) 6 months post transplantation. (HA) FVIII^{-}/$^{-}$. (Gr I) Syngeneic recipient. (GrII) Allogeneic recipient.

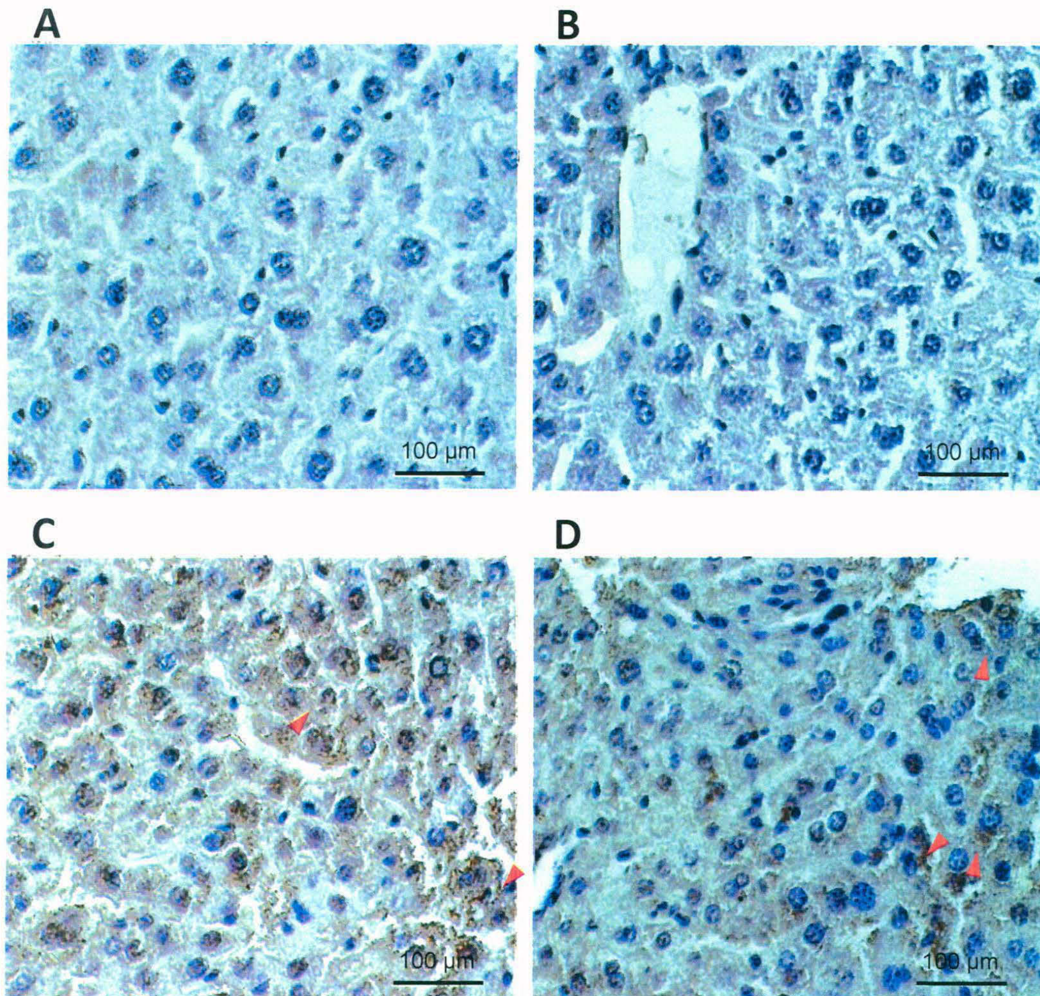
section 4.2.10. As controls, liver sections of WT mouse and HA mouse were also analysed. The results revealed that most of the hepatocytes FVIII heavy chain in WT control liver. However, hepatocytes of HA mouse also were found to express FVIII heavy chain to the same extent. Further analysis using FVIII light chain antibody showed that the light chain is expressed only in WT control liver and not in HA liver. Presence of hepatocytes expressing FVIII light chain protein was also detected in the recipient mice of Group I (Figure 5.10). In order to verify the presence of FVIII in WT LSECs, a different approach was used. Immunofluorescent labeling of FVIII and vWF – which is a marker of LSECs – was performed as described in section 4.2.9. The micrographs showed co-expression of vWF and FVIII confirming the assumption that LSECs also express FVIII protein (Figure 5.11).

Table 5.3: comparison of survival following induced hemorrhage in test and control groups

Test group	Number of mice tested	Number of mice survived	Percentage of survival
C57BL/6J	8	8	100
FVB/J	6	6	100
HA	6	1	16
Group I	6	6	100
Group II	2	0	0

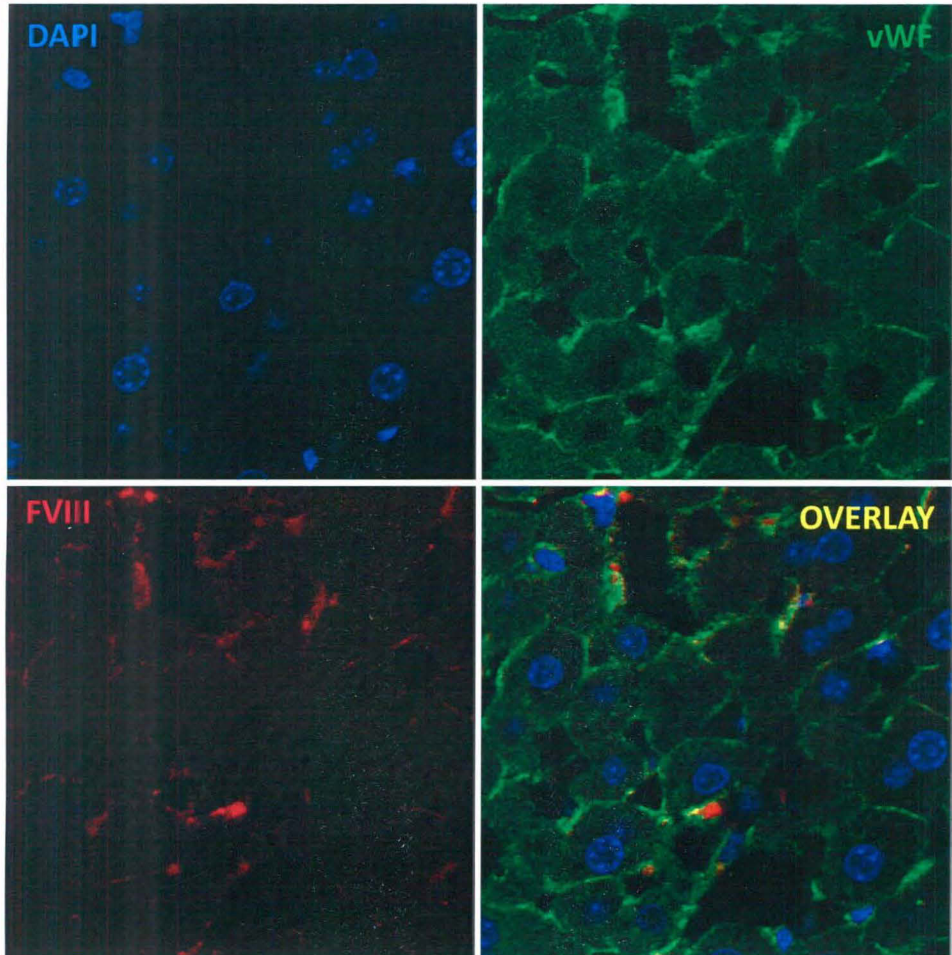
It was unclear whether the FVIII synthesized in the HAT mouse liver originates from donor derived hepatocytes or LSECs. In order to determine this, ultrathin tissue sections of HAT mice liver were examined using transmission electron microscopy. Immunolabeling of FVIII, albumin or vWF was done using different sized gold particles to detect FVIII expressing hepatocytes and LSECs. Colocalization of 40 nm colloidal gold particles bound to FVIII protein

Figure 5.10



Expression of FVIII light chain protein in hepatocytes (representative image). Serial sections of wild type, HA and HAT mouse livers were analyzed by immunohistochemistry. (A) Isotype control in WT mice. (B) FVIII-light chain (lc) staining in HA mice. (C) FVIII-light chain staining in WT mice. (D) FVIII-light chain staining in HAT mice. Red arrowheads show the expression of the FVIII light chain protein. Magnification: 40X.

Figure 5.11



Expression of FVIII light chain protein in LSECs (representative image).

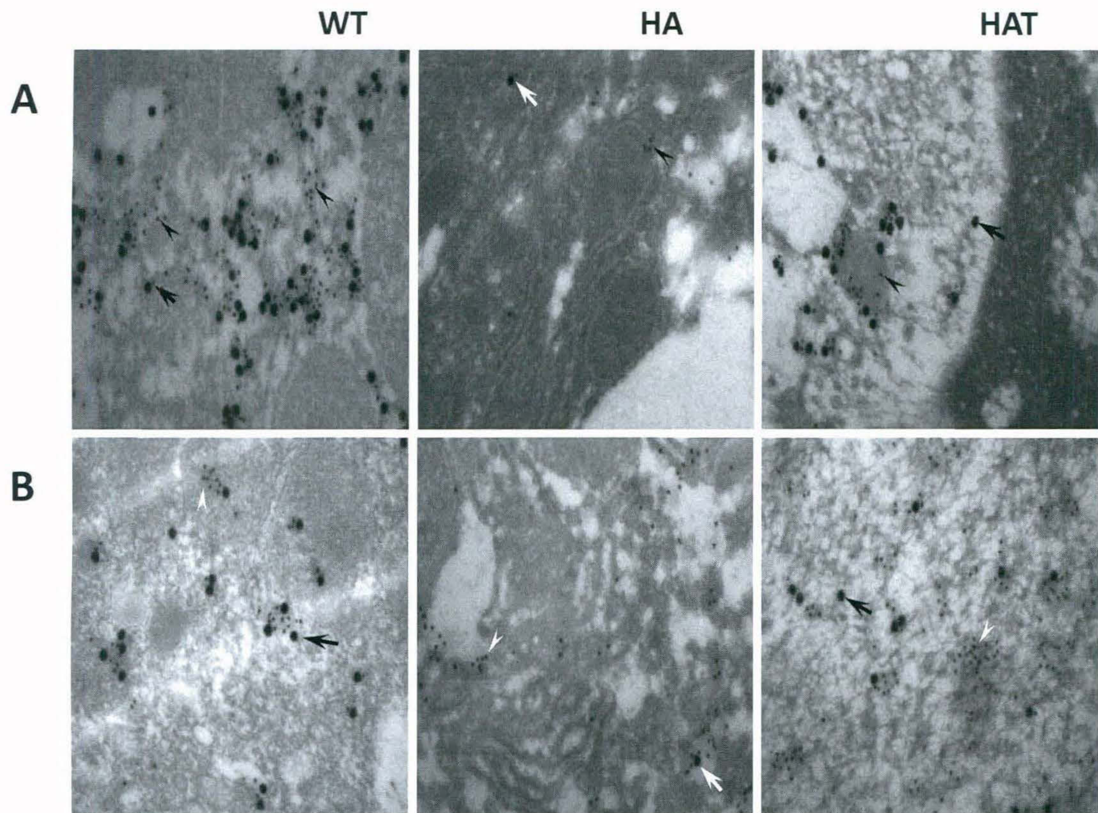
Serial sections of wild type mouse livers were analyzed. Co-expression of vWF and FVIII was identified by immunofluorescence analysis. Magnification: 63X

and 10 nm colloidal gold particles bound to albumin indicated the presence of WT hepatocytes. WT LSECs were identified by colocalization of 40 nm colloidal gold particle (FVIII) and 10 nm colloidal gold particles (vWF) (Figure 5.12). From these results it became clear that transplanted syngeneic WT Lin⁻ BMCs transdifferentiated in to hepatocytes as well as LSECs in the hemophilic mouse liver.

Presence of FVIII protein did not give quantitative information regarding which cell type among the donor derived hepatocytes and LSECs could be considered as the principal source of FVIII in mice undergoing phenotypic correction of hemophilia A. In order to ascertain this, the relative expression of FVIII mRNA in the two cell types was quantified by Real-Time PCR. Firstly, purified fractions of hepatocytes and LSECs were made by digesting WT mouse liver using a two-step collagenase perfusion method to generate a suspension of single cells with viability of 84% (Figure 5.13). Hepatocytes and LSECs were purified out of the cell suspension by following the protocol detailed in section 4.2.15. Both cell populations were stained with albumin and CD31 antibodies. Microscopic examination revealed the presence of very low numbers of hepatocytes in the LSEC population, which indicated that, LSEC population was contaminated minimally. Among the purified hepatocytes, however, there were no detectable CD31 expressing cells indicating that the purity of hepatocyte fraction was absolute (Figure 5.14). The same technique was followed for isolating purified fractions of the two cell types from livers of HAT mice as well. Following this, total RNA was isolated from the two cell populations as described in section 4.2.16 and reverse transcription was carried out. Amplification of a 101 bp amplicon from the A3 domain of *FVIII* using primers designed from exons 16 and 17 of the gene would be possible only if WT mRNA is produced in the liver subject to analysis. The target sequence was amplified in both hepatocyte and LSEC fractions of WT control mice. Similarly, specific amplification was observed in both cell populations of HAT mice. In control HA liver, there was no amplification of *FVIII* gene, whereas low level amplification was seen in control Lin⁻ BMCs. Amplification of *GAPDH* was achieved in all samples (Figure 5.15). These results showed that transcription of the *FVIII* gene happens in both cell types and indicated that the FVIII protein detected immunohistochemically were expressed in these cells and not adsorbed.

Further, Real-Time PCR amplification of the target sequence was carried out as described in section 4.2.18. In HAT mice LSECs and hepatocytes, relative quantification of FVIII expression

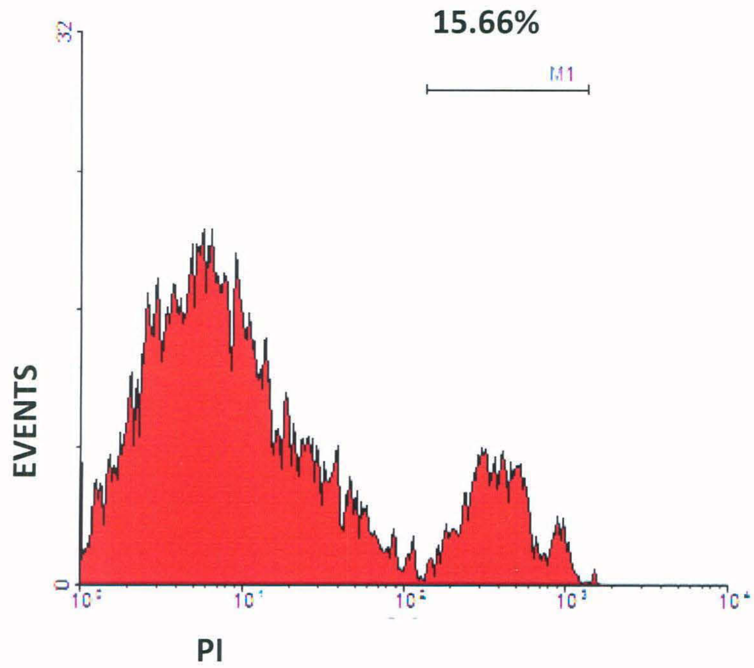
Figure 5.12



Transmission electron microscopy analysis for the expression of FVIII (lc) protein in hepatocytes and endothelial cells (representative image).

Ultrathin sections of liver were labeled with (A) albumin and FVIII (lc) and (B) vWF and FVIII (lc) antibodies and with corresponding two different sizes of gold colloidal particles. Magnification: 82,000X. Albumin (black arrowheads) and vWF (white arrowheads) were detected with 10-nm colloidal gold particles, whereas FVIII (lc) was detected with 40-nm particles (black arrows). White arrows indicate nonspecific binding of FVIII (lc) antibody in HA mice liver section. (HA) FVIII^{-}/$^{-}$. (HAT) syngeneic transplant recipient.

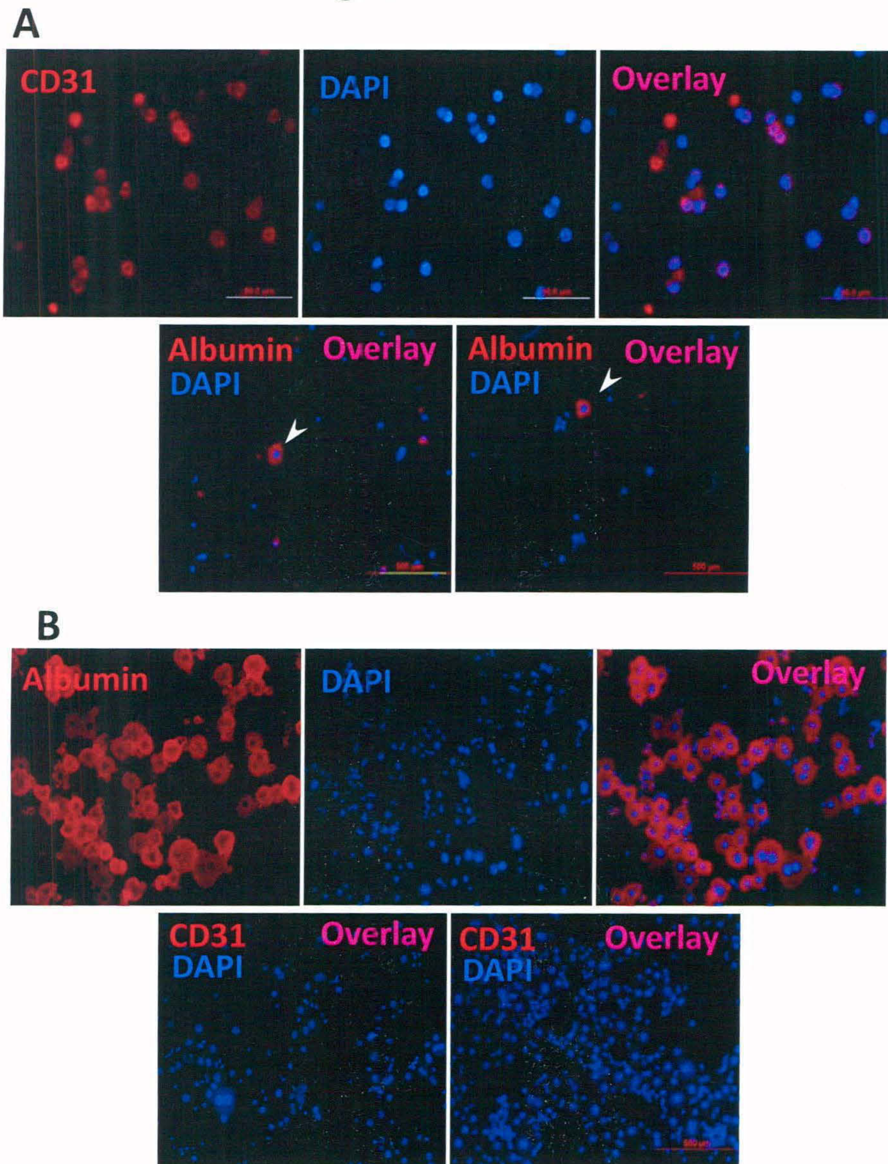
Figure 5.13



Viability of perfusion isolated liver cells.

Single cell preparation of liver cells was made by 2-step collagenase perfusion of liver. Viability was determined by flow cytometric analysis of PI stained cells.

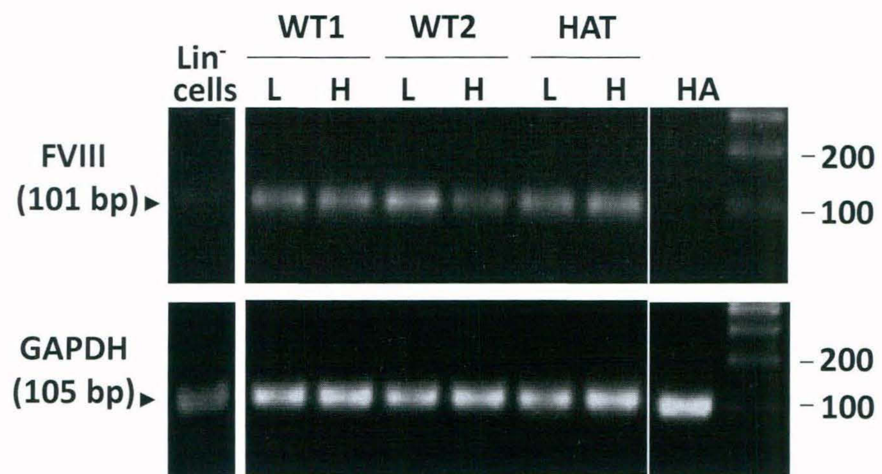
Figure 5.14



Purity of perfusion isolated hepatocytes and LSECs.

Single cell preparation of liver cells was subjected to first differential and later density gradient centrifugations to prepare purified populations of hepatocytes and LSECs. Each fraction was stained with albumin or CD31 antibodies in combination with DAPI in order to determine the level of purity. (A) LSEC fraction. White arrowheads shows contaminating hepatocytes. (B) Hepatocyte fraction

Figure 5.15



mRNA expression of *FVIII* gene in hepatocytes and endothelial cells.
cDNA from WT, HA and HAT mice were amplified using primer pairs designed for Real-time PCR of *FVIII*. *GAPDH* was also amplified as a positive control. The PCR products were resolved in agarose gel electrophoresis.

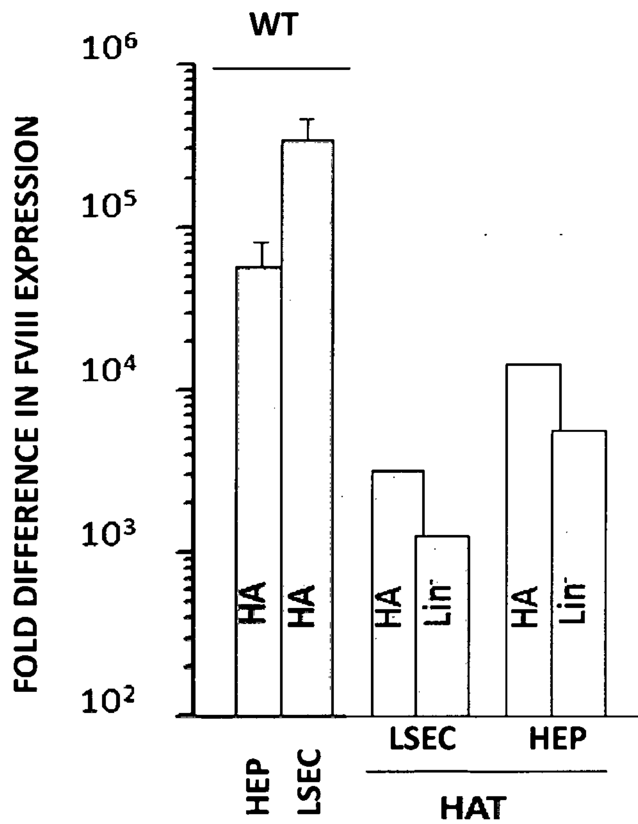
was analysed using two separate calibrators: HA mice liver cDNA and WT mice Lin$^-$ BMC cDNA. Quantification of relative levels of expression of FVIII in hepatocytes and LSECs in WT mice was also done using HA mice liver cDNA as calibrator. The results showed that in a WT mouse, LSECs expressed more FVIII amounting to more than 5 times the expression in hepatocytes. In HAT mice, hepatocytes were found to express 4-5 times more FVIII than LSECs (Figure 5.16).

5.4 Discussion

Replacement of damaged liver by orthotopic liver transplantation serves several functions including a treatment option for genetic or metabolic disorders, if the donor of the liver is genetically normal or WT (Burdelski *et al.*, 1991). In many cases of liver injury, however, replacement of the entire organ is not necessary since the adult liver can regenerate itself following extensive damage. Stem/progenitor cells from bone marrow have been observed to contribute to this process by migration to liver and subsequent trasdifferentiation in to hepatocytes (Piscaglia *et al.*, 2008; Kallis *et al.*, 2007). Indeed, transplantation of stem/progenitor cells has been used for liver repopulation *in vivo* as an effective treatment strategy for liver failure (Sancho-Bru *et al.*, 2009). The potential of bone marrow derived cells to regenerate liver has been demonstrated by a number of researchers (Theise *et al.*, 2000; Lagasse *et al.*, 2000; Schwartz *et al.*, 2002; Jang *et al.*, 2004; Khurana and Mukhopadhyay, 2007; Khurana and Mukhopadhyay, 2008). Transplanted from a WT donor, the BM-derived hepatocytes could replenish the deficient metabolite, thus bringing about a lasting phenotypic correction.

Hemophilia A is an X-linked genetic disorder that affects 1 in 5000 males and consequently has been the subject of long term scientific research. Treatment options currently available for hemophilia A are expensive or intensive. Clinical studies show that approximately 20% of HA patients develop inhibitors to treatment (High, 2006) and that these patients are difficult to treat (Mannucci, 2003). Cell-based therapies using isolated primary hepatocytes (Ohashi *et al.*, 2005; Tatsumi *et al.*, 2008) or LSECs (Do *et al.*, 1999; Follenzi *et al.*, 2008) are suggested to treat clotting disorders. It has been documented that MHC matched WT donor BMC-derived hepatocytes could bring about the desirable phenotypic correction of hemophilia A by synthesis of FVIII in the recipient (Yadav *et al.*, 2009). However, in a clinical

Figure 5.16



Real-time PCR analysis of 101 bp amplicon synthesis by hepatocytes and endothelial cells.

cDNA from wt and HAT mice were analyzed and FVIII expression relative to that of HA or Lin^- donor BMC was calculated. Columns marked HA denotes expression levels in each sample derived by using HA mouse liver cDNA as calibrator. Columns marked Lin^- shows expression levels derived by using Lin^- cell cDNA as calibrator.

set up, transplant rejection will be a major concern since the majority of the donors are likely to be allogeneic with regards to the MHC complex. To establish the feasibility of using MHC mismatched BM-derived progenitor cells in the treatment of hemophilia A, HA mice were subjected to liver damage using acetaminophen, which at high doses induces centrilobular necrosis. In parallel experiments, syngeneic and allogeneic BM-derived progenitor cells (uncommitted BMCs) were transplanted in to the liver-damaged HA mice as described in literature (Yadav *et al.*, 2009). The experimental mice were subjected to analysis either 3 or 6 month post-transplantation. Among the syngeneic recipients, a high percentage of survival was observed at 3 months, which did not reduce significantly till the end of 6 months. However, among the allogeneic recipients very high mortality was observed necessitating the sacrifice of the remaining mice at the first time point in the anticipation of further deaths before the next time point is reached. This high mortality may be attributed to an acute GVHD which is a major cause of mortality in mismatched hematopoietic stem cell transplantations in mice. The donor FVB-GFP mice express the MHC haplotype $H2K^q$ whereas the recipient mice express the $H2K^b$ haplotype. The GVHD that develops in response to a full MHC disparity results in an inflammatory "cytokine storm", which is capable of inducing GVHD in target tissues without the requirement for cognate T cell interaction with MHC on tissue (Teshima *et al.*, 2002). The liver damage induced by injection of acetaminophen might have contributed to the inflammation by promotion of activation and proliferation of inflammatory cells (Reddy and Ferrara, 2009).

The IHC results revealed the presence of donor derived hepatocytes in the centrilobular and other areas of livers in the syngeneic recipient mice 3 months post-transplantation, whereas such cells were completely absent from the livers of the allogeneic recipients. This shows that the allogeneic donor cells were rejected by the host immune system resulting in their failure to assist in regeneration of the damaged liver.

To verify the effect of donor derived hepatocytes on *in vivo* FVIII synthesis in the HAT mice, plasma samples were analyzed using ELISA. The results suggested a positive effect on FVIII synthesis in syngeneic recipients, which could potentially contribute towards phenotypic correction of the disease condition (Yadav *et al.*, 2009). As assumed, there was no FVIII synthesis attributable to donor derived cells in the allogeneic recipient. These observations were further corroborated by the analysis of coagulant activity of the FVIII protein in the HAT

mouse plasma. COATEST results showed significant increase in plasma FVIII activity in syngeneic recipient mice whereas in allogeneic recipient mice no increase was seen.

In humans, hemophilia A has been classified as mild, moderate or severe based on symptoms, which corresponds to the FVIII activity in plasma or amount of FVIII present in plasma. Persons with less than 1% of normal FVIII activity (<0.01 IU/ml) are considered to have severe hemophilia and suffer from spontaneous bleeding, predominantly in joints and muscles. Persons with 1-5% of normal FVIII activity (0.01-0.05 IU/ml) are considered to have moderately severe hemophilia (Tantawy, 2010). They suffer from occasional spontaneous bleeding and severe bleeding following trauma or surgery. Mild hemophilia is characterized by presence of 5-40% FVIII activity in plasma (0.05-0.4 IU/ml) with severe bleeding observed only after major trauma or surgery. However, clinical severity may not always correlate with the *in vitro* assay result (Tantawy, 2010). While it may not be correct to apply the percentages used in classification of hemophilia A in humans to the mouse model, the clinical severity in mouse models may be deduced from the symptoms. In other words, a phenotypic correction of hemophilia in experimental mice can be demonstrated by survival of the mouse after a major trauma which would result in death of untreated HA mice. In order to verify the phenotypic correction of the disease, the experimental mice in both allogeneic and syngeneic recipient groups were subjected to two blood coagulation tests as described previously. The capillary coagulation test showed that average *ex vivo* coagulation time in syngeneic recipient group closely corresponded to that of the WT mice whereas the allogeneic recipient mice presented significantly higher coagulation times. The tail cut challenge test offered the final proof of phenotypic rescue of the experimental mice: the syngeneic recipients registered 100% survival following trauma which was sufficient to kill all of the allogeneic recipients and 84% of the untreated HA mice. These results confirmed the hypothesis that bone marrow derived cells engraft and transdifferentiate to functional hepatocytes in recipient mice livers under syngeneic transplantation settings and fail to do so in allogeneic transplantation settings. Hence, interventions to promote allograft survival in the HAT mice become mandatory in order to effect phenotypic correction of genetic disorders such as hemophilia A. Such an approach would be highly relevant in treatment of hemophilia A in humans, where full MHC compatibility between donor and recipient is highly improbable.

FVIII is majorly synthesized in liver. It has been demonstrated by various researchers that the cellular source of FVIII is hepatocytes or LSECs or both. Indeed, transplantation of LSECs has been shown to be effective in correction of hemophilia A phenotype in mice (Kumaran *et al.*, 2005; Follenzi *et al.*, 2008). These studies were in contradiction to a previous study, which demonstrated that human hepatocytes transplanted under the kidney capsules of mice render therapeutic benefit (Sarkar *et al.*, 2000). Kumaran *et al.* (2005) transplanted hepatocytes grown on Cytodex-3 microcarriers in the peritoneal cavity and showed that the transplanted hepatocytes engrafted in the cavity remained viable and functional for 1 week. However, hepatocytes did not produce phenotypic correction in HA mice, whereas LSECs did. Although earlier reports (Demetriou *et al.*, 1986; Gupta *et al.*, 1994) showed that the expression of some proteins was maintained by hepatocytes for more than a week in the peritoneal cavity, the same may not be true in the case of a complex protein like FVIII. The ability of hepatocytes to produce FVIII may have been lost in the peritoneal cavity, because they were removed from their natural hepatic environment. This loss of FVIII production may not be the case with LSECs, because endothelial cells occur naturally in all tissues. Also, the failure to express FVIII by hepatocytes (Kumaran *et al.*, 2005) may be due to the short duration of experiments. Further, the tolerogenic properties of the liver may have an important role in the expression of FVIII by hepatocytes in HA mice; these properties are lacking in the peritoneal cavity (Selden *et al.*, 1995; Lau *et al.*, 2003; Crispe, 2003). It would therefore, be interesting to find out the relative contribution of the two cell types to FVIII synthesis in the HAT mouse liver. IHC results showed that hepatocytes expressed intact FVIII light chain in WT and HAT mice livers. Immunofluorescent studies showed FVIII expression in LSECs as well. Further, electron microscopic analysis of HAT mice livers revealed expression of FVIII in hepatocytes and LSECs. Quantification of *FVIII* expression in the two cell types in HAT mice livers using Real-Time RT-PCR showed that hepatocytes expressed more *FVIII* gene after liver damage and reconstitution, which was different from the scenario in WT mice in which, LSECs were found to be the major source of FVIII. This difference is inexplicable with the currently available data and further extensive research involving transplantation of LSECs and hepatocytes in to different groups of mice may be required in order to arrive at definite conclusions.

Study of engraftment and functional modulation of liver in HA mice by allogeneic BM-derived cells

6.1 Introduction

Hemophilia A is an X-linked genetic disorder which could be treated through various approaches mostly aimed at replacement of the absent functional FVIII protein. These include introduction of the gene, protein, or the cells capable of synthesizing the protein. Gene therapy aims to introduce the functional gene through viral vectors or naked plasmids and envisages continuous supply of FVIII in the recipient animal through highly reproducing stem cells or long lived cells like muscle cells or neurons (Rick *et al.*, 2003; Kootstra *et al.*, 2003; High, 2006; Porada *et al.*, 2011). Infusion of FVIII concentrates was found to be an effective strategy in more than 82% of the target population (Powell *et al.*, 2000; Nemes *et al.*, 2007). Transplantation of hepatocytes (Ohashi *et al.*, 2005) or LSECs (Follenzi *et al.*, 2008) or whole liver (Gordon *et al.*, 1998; Ashrani *et al.*, 2004) could result in therapeutic correction of hemophilia A. However, counterproductive traits such as development of inhibitory antibodies, high cost of treatment and need for lifelong infusions, associated with these treatment strategies (Green, 2011) makes it imperative that further research be performed with the intention of avoiding development of inhibitory antibody production in the patient as well as keeping the cost and complexity of the therapy to a minimum. It was observed in the earlier study that transplantation of BM-derived stem cells in a liver-damaged mouse leads to permanent engraftment and differentiation of these cells to hepatocytes and LSECs, both of which are capable of secreting functional FVIII protein (Yadav *et al.*, 2011). It has been reported that there was no development of inhibitor antibody till 18 months after transplantation in this model (Yadav *et al.*, 2009) which was presumably due to the 'liver tolerance effect', which mediates local and systemic tolerance to self and foreign antigens. This phenomenon has been attributed to non-parenchymal resident cells such as dendritic cells, LSECs, Kupffer cells as well as hepatic stellate cells expressing anti-inflammatory mediators and inhibitory cell surface ligands for T cell activation (Tiegs and Lohse, 2010). However, such success with BMC transplantation was restricted to the syngeneic setting as shown earlier in the present study. Allograft rejection and GVHD present two of the greatest huddles in clinical transplantation. Thus, development of efficient methods to ensure stable engraftment and differentiation of allogeneic BM-derived cells is necessary to arrive at a reliable treatment strategy for hemophilia A. In the present study,

immunomodulation was induced by using dexamethasone, generation of donor-specific macrochimerism or regulatory T cells.

6.2 Methods

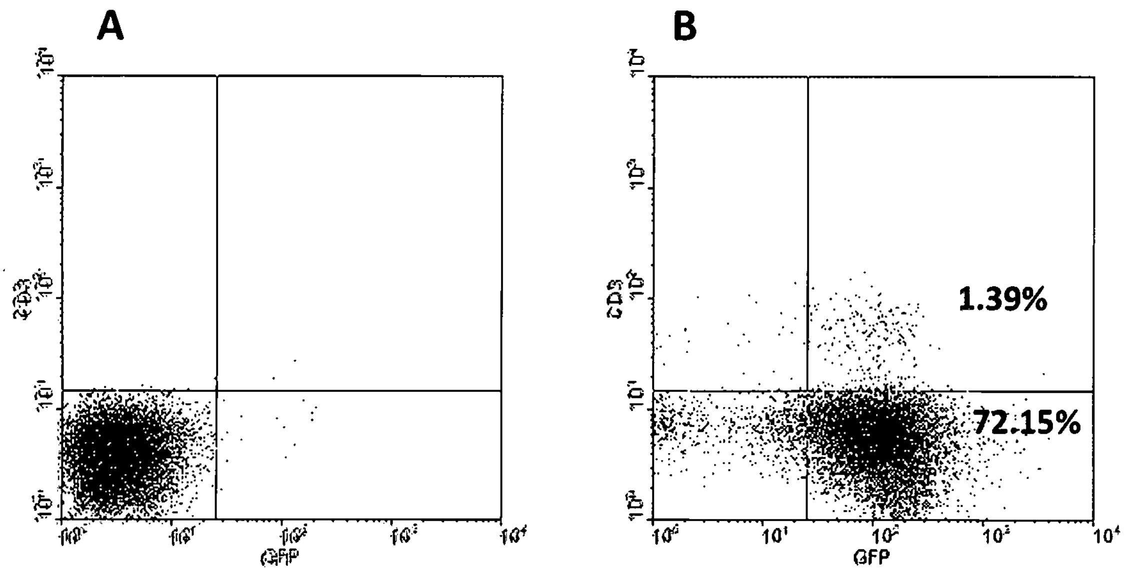
Three groups of 6-8 weeks old HA mice were used in this study. One group was lethally irradiated and bone marrow reconstituted with FVB/J mouse bone marrow to create chimeric mice. The second group was treated with dexamethasone to induce immune suppression. The third group was not treated. Acute liver injury was induced in the HA mice and Lin^- BMCs isolated from FVB-GFP ($H2K^q$) mice were transplanted in the experimental groups. In the third group, T_{reg}s isolated from HA mice and sensitized *in vitro* towards $H2K^q$ antigen were also transplanted along with Lin^- BMCs. Three months after transplantation, the mice were bled and plasma was collected and stored frozen. The plasma samples were subjected to analysis by ELISA for detection and quantification of FVIII protein. Further, the *in vitro* FVIII activity in the plasma samples was determined using COATEST®. To detect efficiency of blood clotting, blood coagulation time of transplant-recipient mice was determined by capillary coagulation method. To determine the survival after trauma, tail clip challenge was conducted. The mice were sacrificed and presence of GFP expressing hepatocytes in liver was investigated by immunofluorescence of cryosections. FVIII expression in recipient mice liver was analyzed by RT-PCR.

6.3 Results

6.3.1 Lethal irradiation followed by BM-transplantation leads to peripheral blood chimerism in HA mice

To establish BM chimerism, groups of recipient HA mice were lethally irradiated at a dose of 960 cGy and transplanted with 10×10^6 unfractionated BM cells from FVB/J or FVB-GFP mice. To demonstrate development of chimerism, peripheral blood from FVB-GFP BMC transplanted mice was collected and $CD3^+$ cells were analyzed by flow cytometry for expression of GFP. The results showed that approximately 74% of PBMCs were donor-derived. Among the PBMCs, 1.39% was donor derived T cells (Figure 6.1). This indicated that a donor-specific macrochimerism has been established in the recipient HA mice. However,

Figure 6.1



Demonstration of peripheral blood chimerism in BMT-HA

HA mice were lethally irradiated [960 cGy] and transplanted IV unfractionated bone marrow cells of FVB-GFP. One month later, PBMCs were stained for CD3 and flow cytometrically analyzed for the presence of GFP-expressing T cells. (A) Unstained HA mouse PBMCs. (B) PBMCs from BMT-HA mouse.

the chimeric mice (BMT-HA) showed poor survival ability. One month after transplantation, the survival was found to be 88% which dropped to 6% (one out of 17 mice) by the end of 3 months after transplantation (Figure 6.5). The group could not be analyzed and the approach was abandoned.

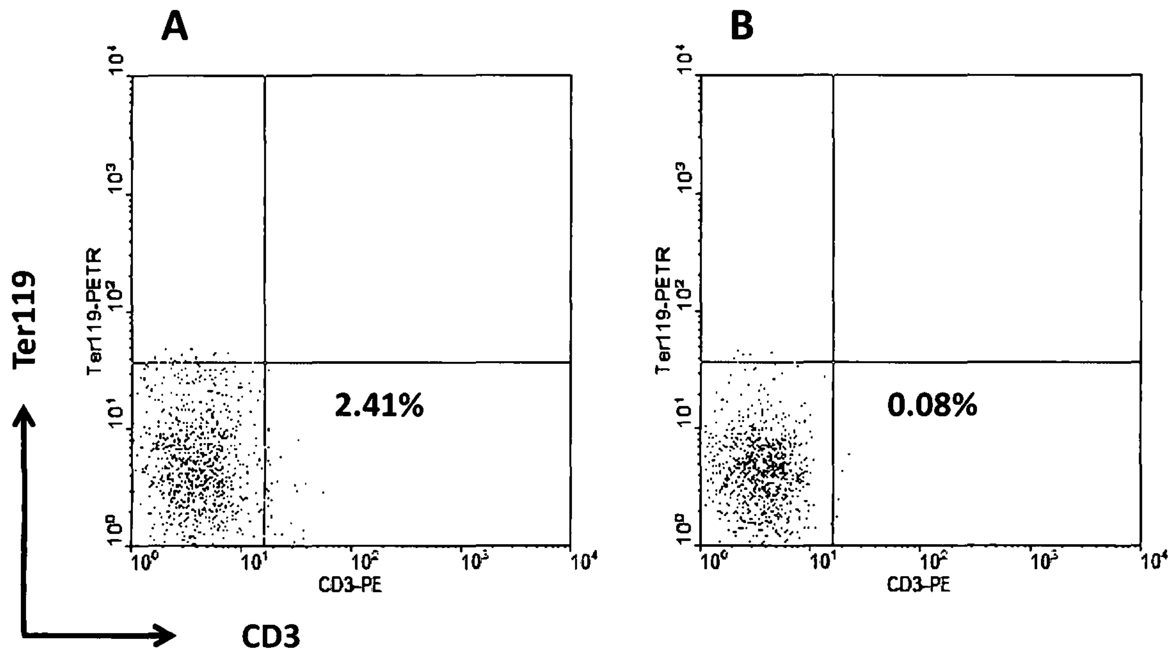
6.3.2 Dexamethasone causes immune suppression in HA mice

The next approach to prevent allograft rejection was to induce immune suppression using dexamethasone, an immunosuppressive agent. Peripheral blood from the experimental mice was collected one week after the commencement of treatment and $CD3^+$ cell count was analyzed by flow cytometry. The result showed a reduction of T cell count from 2.4% to 0.8% (Figure 6.2). This indicated that the mice may not be able to mount a successful T cell dependent immune response. The mice were subjected to liver damage by administration of acetaminophen and were transplanted with 0.25×10^6 Lin^- BMCs from FVB-GFP mice. The immunosuppressive regimen was continued for the length of the experiment. Three months post-transplantation, survival in the group was only 33% (3 mice out of 9) (Figure 6.5). The remaining mice were sacrificed at 3 months time and analyzed for donor derived hepatocytes. Evaluation of phenotypic indicators of hemophilia A was carried out.

6.3.3 T_{reg}s suppress T_h cell activity

$CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ T_h cells were isolated by FACS (Figure 6.3). T_{reg} mediated suppression of polyclonal T cell response was determined as described in section 4.2.7.2. Flow cytometric analysis of CFSE labeled T_h cells showed that following polyclonal activation, 46.5% of the cells undergo divisions and therefore exhibit CFSE dilution. Addition of T_{reg}s decreased the dividing cell percentage. The reduction in cell division was in proportion to the T_{reg} cell concentration in the mixed lymphocyte reaction (MLR) (Figure 6.4). Following this, freshly isolated T_{reg}s were sensitized towards donor cell antigen as described in section 4.2.7.3 and were transplanted along with 0.25×10^6 Lin^- BMCs from FVB-GFP mice. The survival of mice in the three groups of HA mice in which T_{reg}s were transplanted, was found to be the highest among all allotransplantation groups (Figure 6.5). At 3 months post-transplantation, the mean survival percentage of the T_{reg} groups was significantly higher ($p<0.05$) than that of the immunosuppressed group, the chimera group and the

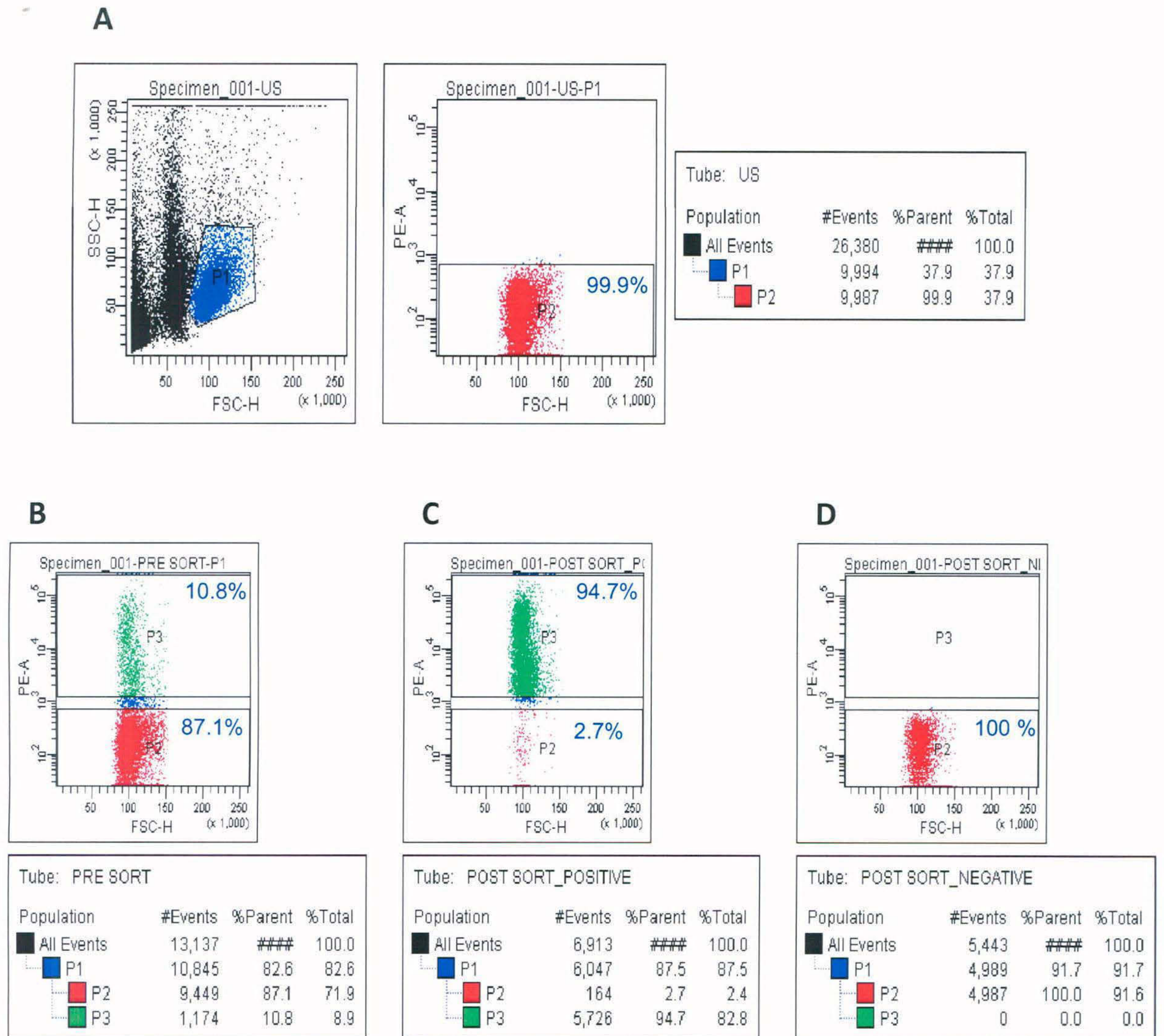
Figure 6.2



Dexamethasone mediated immune suppression

Immunosuppression was induced by IM injection of dexamethasone. One week after the commencement of treatment, PBMCs were stained for CD3 and flow cytometrically analyzed for the presence of T cells. (A) Untreated HA mouse PBMCs. (B) Dexamethasone treated HA mouse PBMCs.

Figure 6.3



Tube: US

Population	#Events	%Parent	%Total
All Events	26,380	####	100.0
P1	9,994	37.9	37.9
P2	9,987	99.9	37.9

Tube: PRE SORT

Population	#Events	%Parent	%Total
All Events	13,137	####	100.0
P1	10,845	82.6	82.6
P2	9,449	87.1	71.9
P3	1,174	10.8	8.9

Tube: POST SORT_POSITIVE

Population	#Events	%Parent	%Total
All Events	6,913	####	100.0
P1	6,047	87.5	87.5
P2	164	2.7	2.4
P3	5,726	94.7	82.8

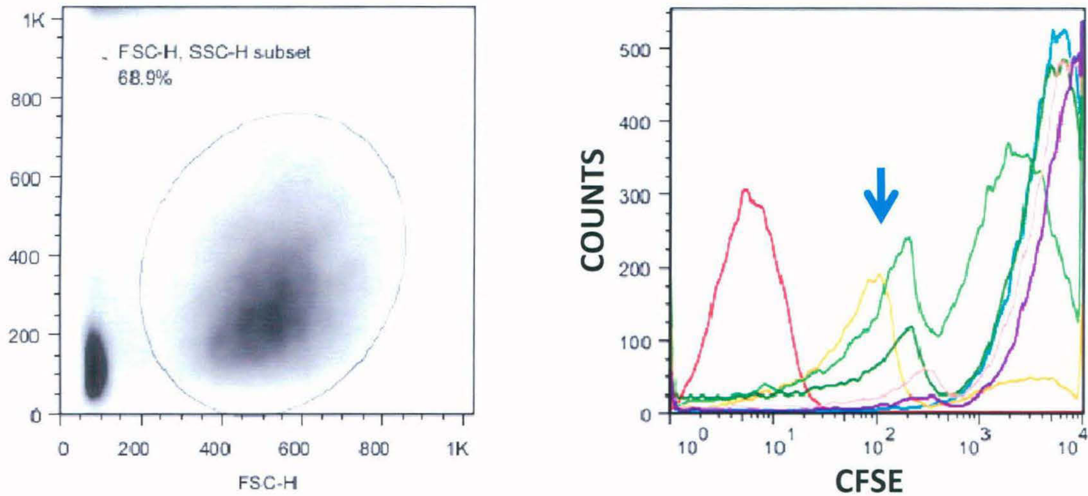
Tube: POST SORT_NEGATIVE

Population	#Events	%Parent	%Total
All Events	5,443	####	100.0
P1	4,989	91.7	91.7
P2	4,987	100.0	91.6
P3	0	0.0	0.0

Isolation of T regs

$CD4^+$ HA splenocytes were isolated by MACS, labeled with CD25-PE and separated in to T_{reg} and T_h populations by FACS in a FACSAria III Cell Sorter. (A) Unstained splenocyte population. (B) $CD4^+CD25^{+/-}$ population. (C) $CD4^+CD25^+$ T_{reg}s. (D) $CD4^+CD25^-$ T_h cells.

Figure 6.4

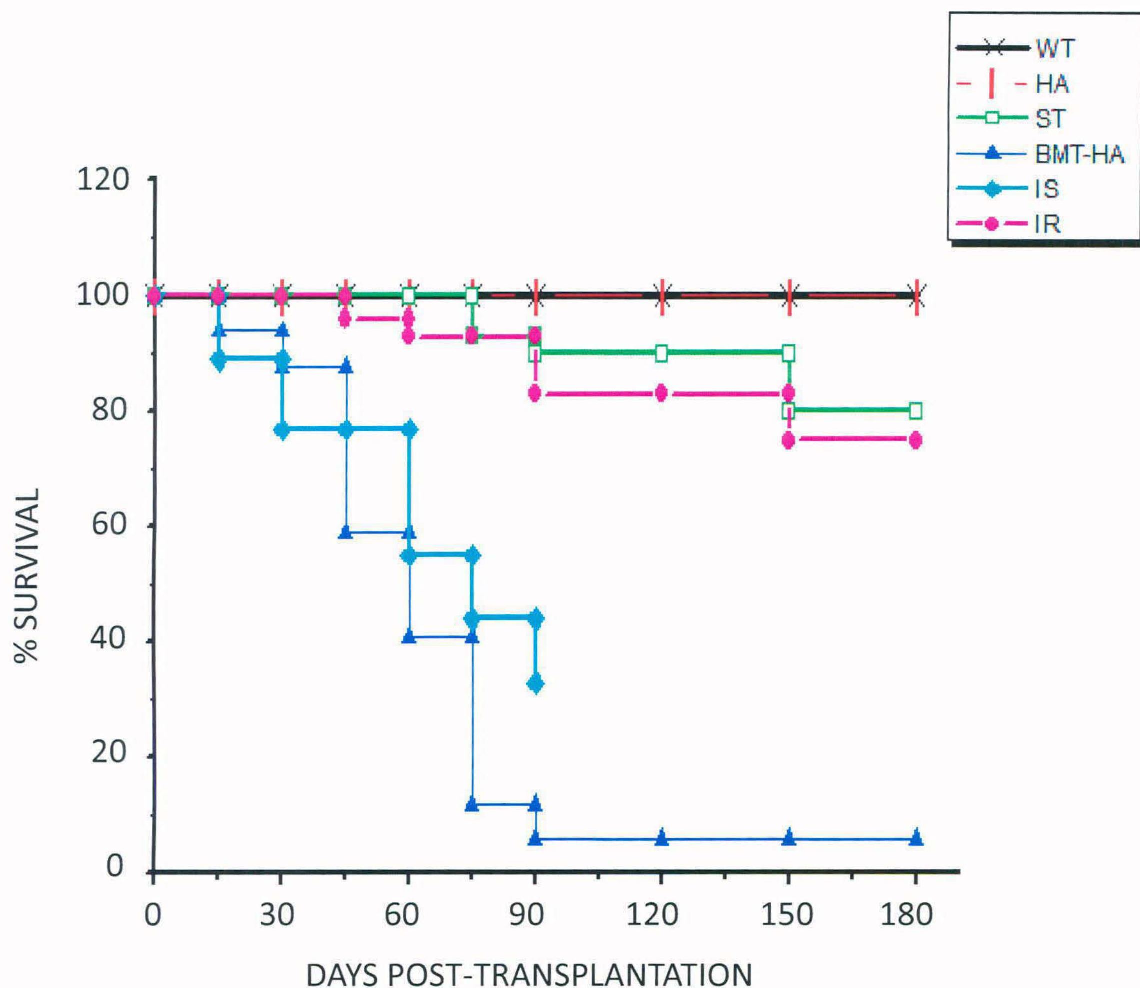


Sample		Ratio	% dividing cells
CFSE negative population	(red line)	---	---
CFSE labeled T_h population	(blue line)	---	---
Polyclonal activation	(orange line)	---	46.5
T_h : T_{reg}	(green line)	25:1	21.8
T_h : T_{reg}	(dark green line)	12.5:1	13.5
T_h : T_{reg}	(pink line)	6.25:1	4.25
T_h : T_{reg}	(purple line)	3:1	2.68

Inhibition of CFSE dilution by Tregs

CFSE labeled, PHA stimulated T_h cells were cultured in vitro along with T_{reg}s at varying ratios. After 4 days, the dilution of CFSE in ratio was analyzed by flow cytometry. The arrow denotes cells that have undergone dilution.

Figure 6.5



Kaplan-Meier survival graph of experimental and control groups

% survival of BMT-HA, IS and IR groups over a period of 6 months was plotted along with ST, WT and HA control groups. BMT-HA group was abandoned at 3 months after BMT and the surviving mice in IS group was sacrificed at 3 months post-transplantation.

allotransplantation control group (Table 6.1), but not significantly lower than the survival percentage of the syngeneic transplantation group ($p>0.05$). One of the T_{reg} groups was retained till the end of 6 months when they were subjected to analysis. The survival percentage among this group was close to that of the syngeneic transplantation group at the same time point (Table 6.1).

Table 6.1: Comparison of percentage of survival among the experimental groups following transplantation/treatment

Experimental group	Survival percentage at 3 months	Survival percentage at 6 months
Syngeneic transplantation control	93%	80%
Allogeneic transplantation control	33%	-
Immunosuppressed group	33%	-
Radiation chimera group	6%	-
Immunoregulation group	85%	75%

6.3.4 Immunosuppression and immunoregulation results in successful engraftment of allogeneic BM progenitor cells in recipient mouse livers

Analysis of the three groups of immunoregulated mice (referred to as IR1, IR2 and IR3) and immunosuppressed mice (referred to as IS) was carried out 3 months post-transplantation. IR2, IR3 and IS groups were sacrificed at that time point for detailed analysis. IR1 group was maintained till the end of 6 months post-transplantation before the next round of analysis. In order to determine the efficiency of immunosuppressive therapy using dexamethasone and

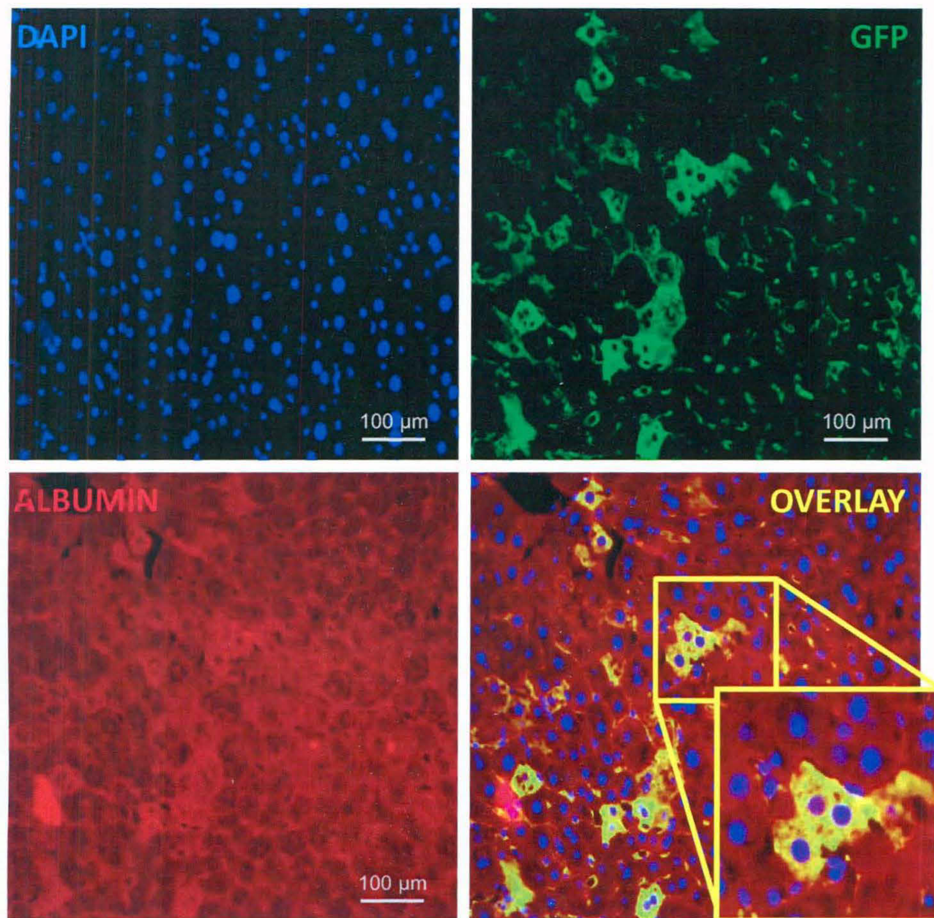
immune tolerance induction using T_{reg}s in facilitating engraftment and transdifferentiation of allogeneic BM derived cells in HA mouse liver, immunohistochemical analysis of liver sections was carried out. Donor derived hepatocytes were found to be present in the livers of IR1, IR2 and IR3 mice (Figure 6.6) and IS mice (Figure 6.7), based on coexpression of albumin and GFP. These results were comparable with that of syngeneically transplanted (ST) mice described previously.

6.3.5 Engrafted BMCs synthesize and secrete full length FVIII protein into recipient mice plasma

RT-PCR analysis of recipient mice livers was carried out to determine expression of WT donor-derived *FVIII* mRNA. The results, which were comparable to those obtained for ST mice and WT mice, showed that IR1, IR2, IR3 and IS mice WT *FVIII* mRNA (Figure 6.8). Further, mice in all experimental groups were bled and plasma was collected. In order to further assess the functionality of BM-derived hepatocytes in recipient mouse liver, plasma FVIII concentration was analyzed using ELISA. The normalized absorbance of pooled WT mouse plasma sample reactions was taken as 100% and the percentages of plasma FVIII expression of HAT mice samples were calculated as % WT plasma FVIII concentration. The results showed that all IR (T_{reg}) groups showed a consistent increase in plasma FVIII concentration which was not significantly lower than the increase in plasma FVIII concentration in ST mice. IS mice also showed an increase in plasma FVIII concentration. These changes were not statistically significant ($p>0.05$) when compared to the increase in plasma FVIII concentration in ST mice. Six months post-transplantation, the plasma FVIII concentration increased slightly in IR1 group and was at comparable levels with the values for ST group (Figure 6.9). The results are summarized in Table 6.2.

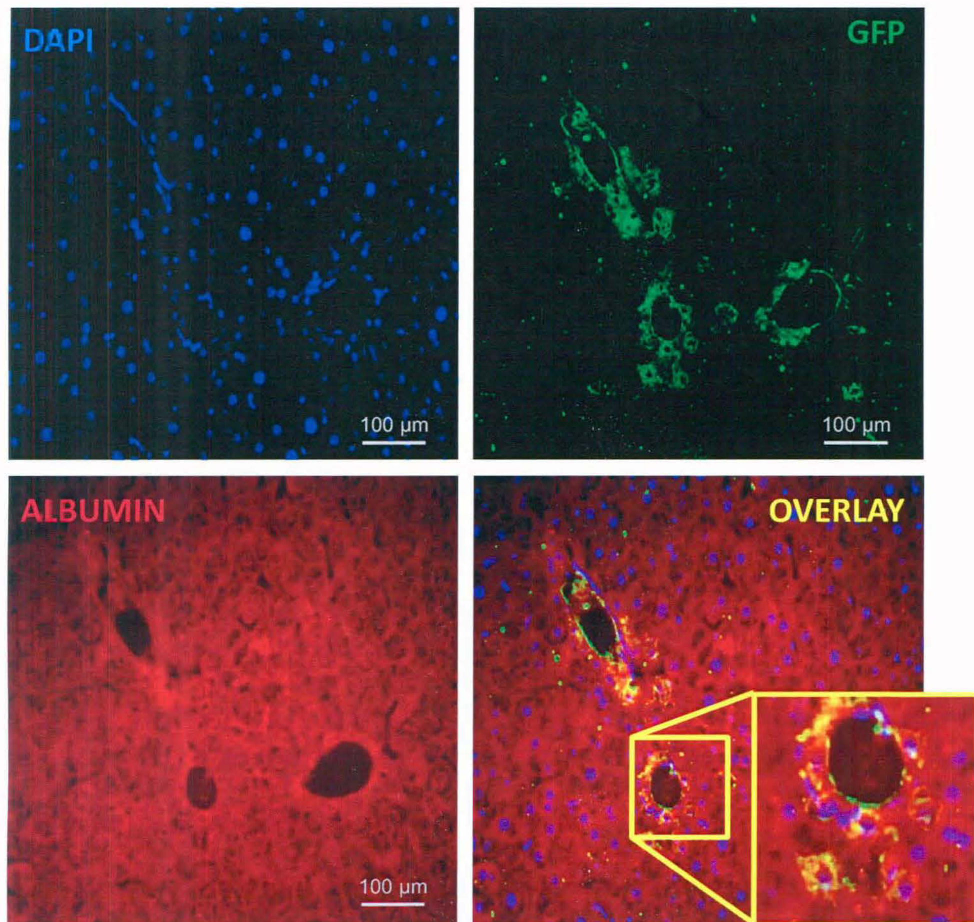
These results indicated that there will be a rise in plasma FVIII activity as well. To test this possibility, the plasma samples were analyzed using COATEST®. The results are summarized in Table 6.3. Predictably, the *in vitro* FVIII activity increased in all the experimental groups, and was not significantly different from that of ST group ($p>0.05$) at 3 months post-transplantation. This was followed by a further marginal increase in FVIII activity in IR1 group

Figure 6.6



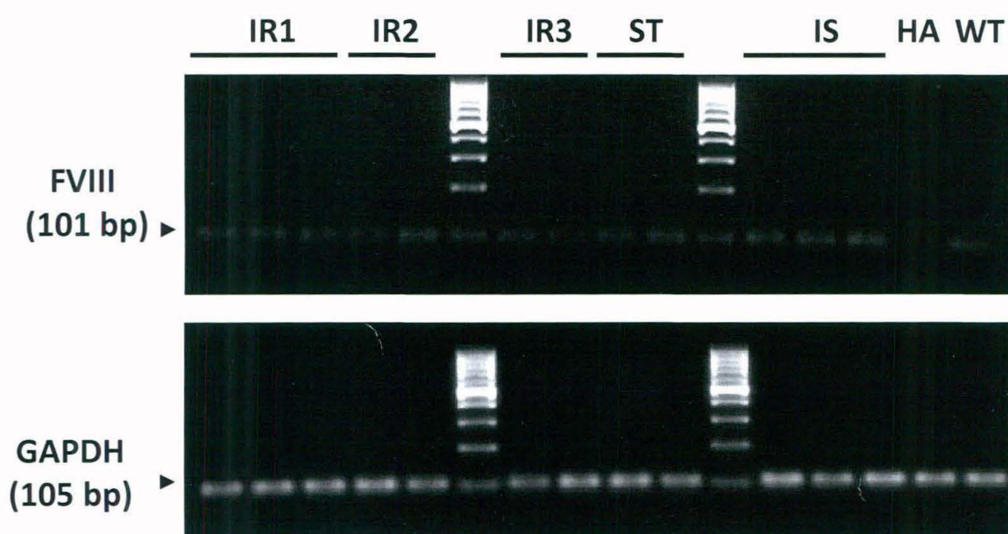
Coexpression of GFP and albumin in IR mouse liver (representative images).
HA mice were transplanted with allogeneic uncommitted BMCs along with sensitized HA-derived T_{reg}s. Liver sections were stained for GFP (Green) and albumin (Red) for the presence of donor derived hepatocytes. Magnification: 20X

Figure 6.7



Coexpression of GFP and albumin in IS mouse liver (representative image).
HA mice under dexamethasone treatment were transplanted with allogeneic uncommitted BMCs. Liver sections were stained for GFP (Green) and albumin (Red) for the presence of donor derived hepatocytes. Magnification: 20X

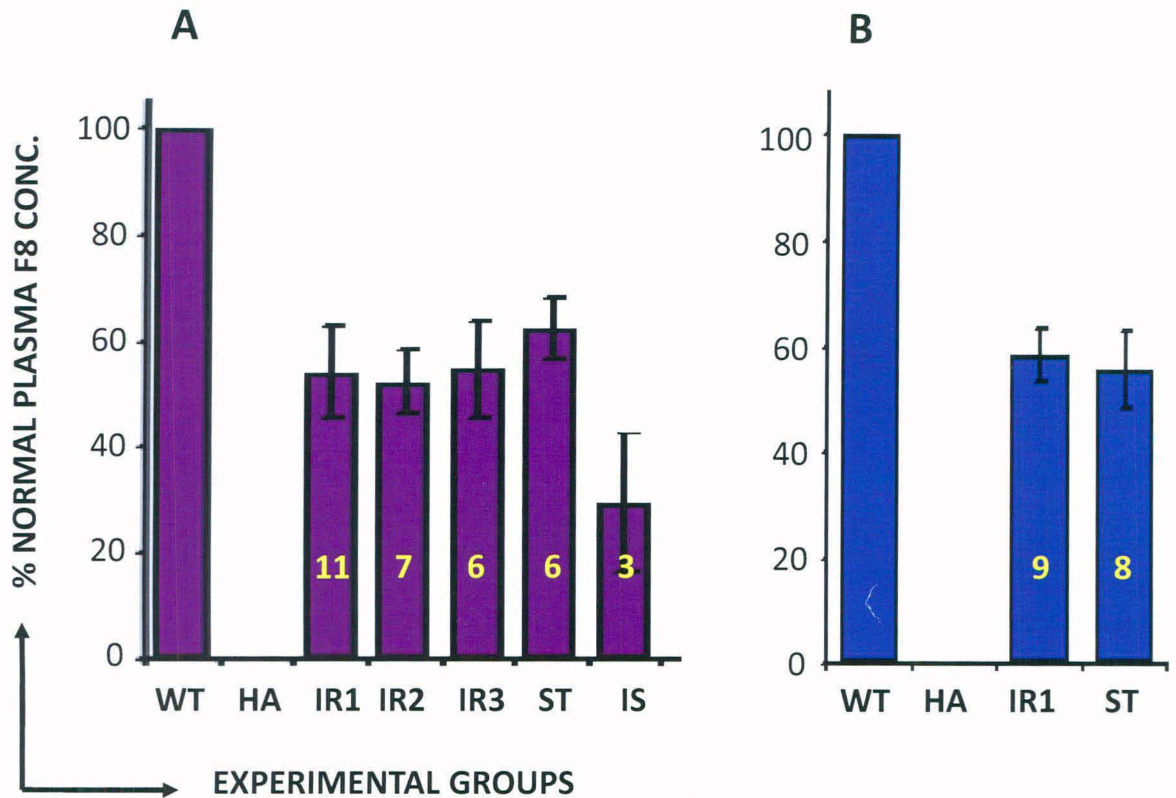
Figure 6.8



Expression of *FVIII* mRNA in recipient and control mouse livers

Mice belonging to each group were sacrificed and total liver mRNA was reverse transcribed. A 101 bp amplicon of FVIII A3 domain was amplified by PCR. *GAPDH* was amplified as a positive control. (IR1 to IR3) T_{reg} transplanted groups. (ST) Syngeneic transplantation group. (IS) Dexamethasone group. (HA) FVIII^{-}/$^{-}$. (WT) FVB/J.

Figure 6.9



Rise in plasma FVIII concentration following transplantation

Plasma collected from experimental and control mice was analyzed for FVIII protein concentration using ELISA. WT mouse plasma FVIII concentration was taken as 100% and values for HAT mice were expressed as % wild type plasma FVIII concentration. **(A)** 3 months post-transplantation. **(B)** 6 months post transplantation. (IR1 to IR3) T_{reg} transplanted groups. (ST) Syngeneic transplantation group. (IS) Dexamethasone group. (HA) FVIII^{-}/$^{-}$. (WT) FVB/J. Number of mouse in each group was mentioned in the respective bars.

Table 6.2: Change in FVIII concentration in recipient mice plasma under different transplantation settings.

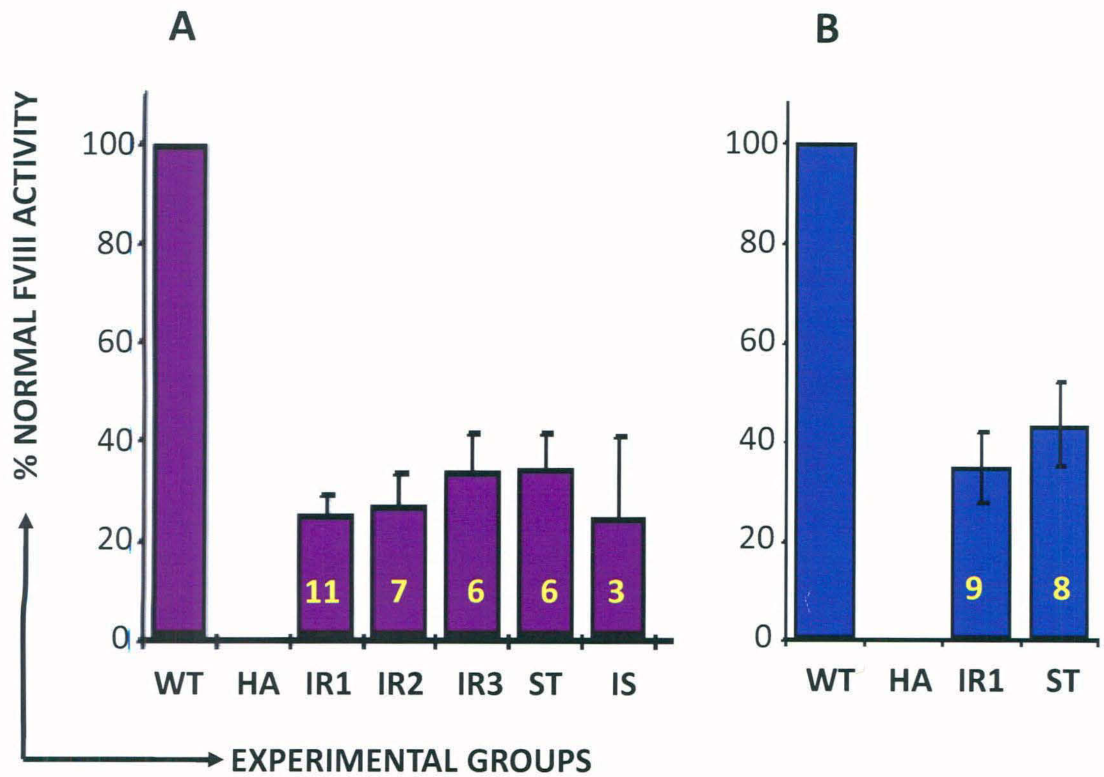
Experimental group	FVIII concentration 3 months post-transplantation (% WT)	FVIII concentration 6 month post-transplantation (% WT)
IR1	54.3 ± 8.7	58.4 ± 5
IR2	52.5 ± 6	-
IR3	55.1 ± 9.1	-
ST	62.62 ± 5.9	55.8 ± 7.3
IS	29.6 ± 13	-

by the end of 6 months after transplantation. This observation closely resembled the results obtained in ST group. There was no significant difference in the magnitude of *in vitro* plasma FVIII activity between the two groups (Figure 6.10).

6.3.6 Donor derived hepatocytes contribute to correction of hemophilia A phenotype

Earlier it was established that syngeneically transplanted BMCs could give rise to functional hepatocytes in a hemophilic recipient and improve the coagulation efficiency of blood by production of functional FVIII protein. However, in allogeneic setting, the donor BMCs failed to engraft. In this study, it was found that using immunosuppressive or tolerogenic methods, engraftment and differentiation of BMCs into functional hepatocytes can be ensured. Further experiments to ascertain the phenotypic correction achievable through these methods were conducted by *ex vivo* whole blood coagulation time test and tail cut challenge.

Figure 6.10



Rise in *in vitro* plasma FVIII activity following transplantation

Experimental and control mice plasma was subjected to COATEST®. WT mouse plasma FVIII activity was taken as 100% and values for experimental mice were expressed as % WT plasma FVIII activity. **(A)** 3 months post-transplantation. **(B)** 6 months post transplantation. (IR1 to IR3) T_{reg} transplanted groups. (ST) Syngeneic transplantation group. (IS) Dexamethasone group. (HA) FVIII^{-}/$^{-}$. (WT) FVB/J. Number of mouse in each group was mentioned in the respective bars.

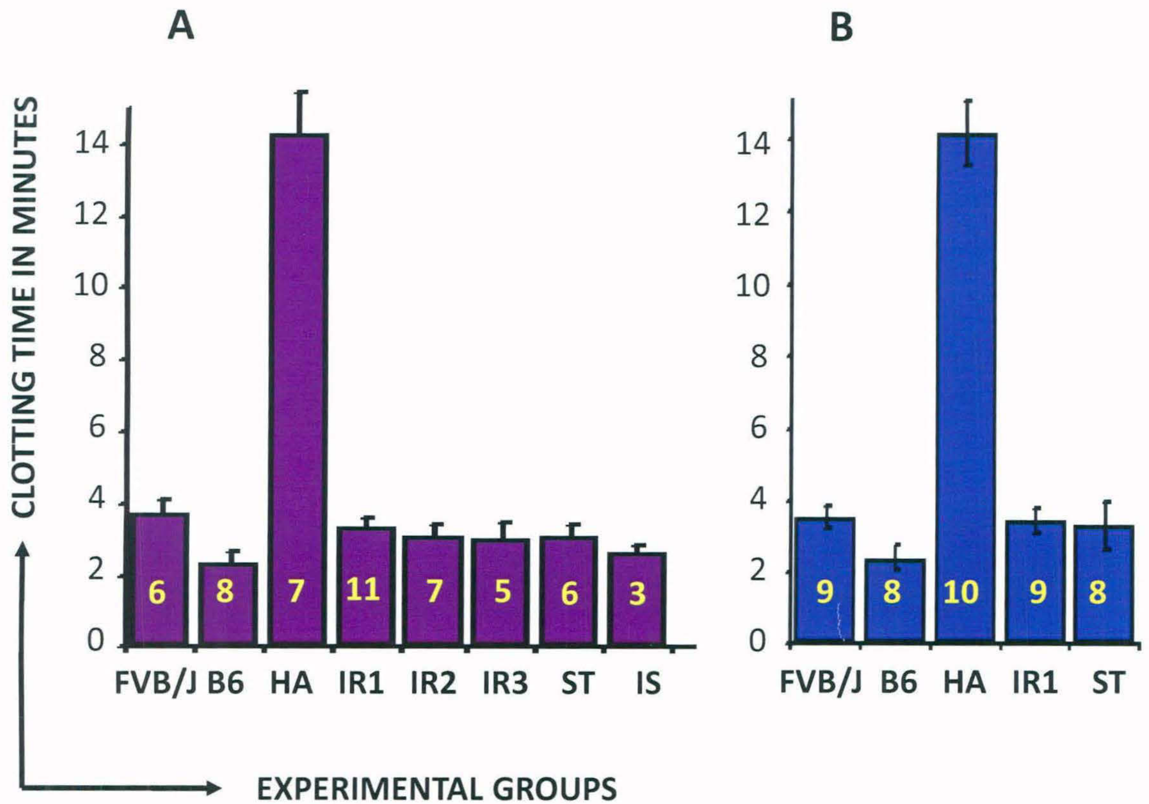
Table 6.3: Change in FVIII activity in recipient mice plasma under different transplantation settings.

Experimental group	FVIII activity 3 months post-transplantation (% WT)	FVIII activity 6 month post-transplantation (% WT)
IR1	25.2 ± 4	34.8 ± 7.4
IR2	27.4 ± 6.4	-
IR3	34.0 ± 7.4	-
ST	34.5 ± 6.9	43.5 ± 8.7
IS	24.8 ± 16.3	-

Coagulation time of blood drawn in capillaries was measured in minutes for each group of experimental mice and compared with the coagulation time shown by the WT mice. The results are summarized in Table 6.4. At 3 months post-transplantation, all experimental groups showed significantly lower coagulation time when compared to that of HA mice. These values closely corresponded to the coagulation times observed for the WT mice. The observed reduction in coagulation time remained consistent at the end of 6 months post-transplantation for the groups tested (Figure 6.11). There was no significant difference between the coagulation times of any of the experimental groups and the coagulation time of ST group at either time point.

In order to assess the impact of the increased coagulation efficiency in hemophilia A phenotype, the mice were subjected to tail cut challenge. Following amputation of tail tips 3 months post-transplantation, 100% of the IR1, IR2, IR3, IS, ST and WT mice survived, whereas 86% of the hemophilic mice died within 24 hours. The therapeutic correction of hemophilia A

Figure 6.11



Effect of increased plasma FVIII activity in ex vivo blood coagulation time

Experimental and control mice were subjected to capillary clotting time test. Clotting time in minutes was compared with that of HA and corresponding WT mice. **(A)** 3 months post-transplantation. **(B)** 6 months post transplantation. (IR1 to IR3) T_{reg} transplanted groups. (ST) Syngeneic transplantation group. (IS) Dexamethasone group. (HA) $FVIII^{-}/^{-}$. (WT) FVB/J. (B6) C57BL/6J. Number of mouse in each group was mentioned in the respective bars.

was found to be sustained till the end of 6 months in the IR1 group as repetition of tail cut challenge once again resulted in 100% survival over 24 hours (Figure 6.12).

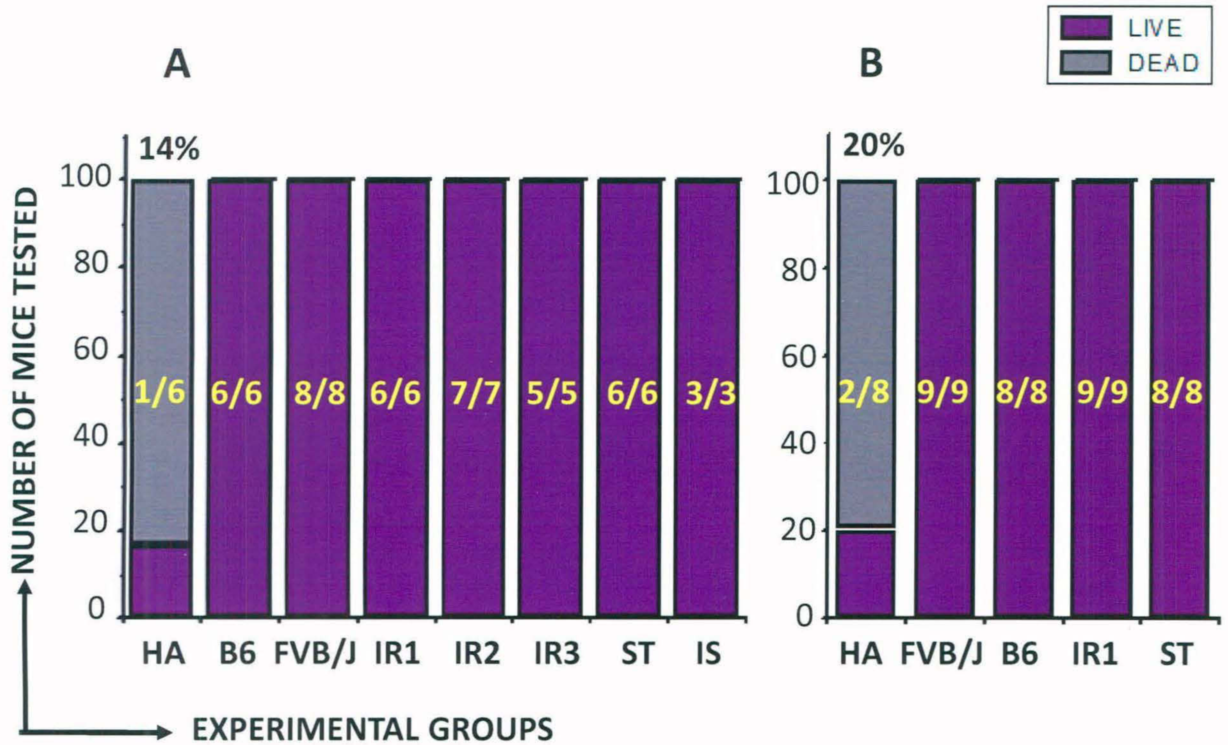
Table 6.4: Reduction in *ex vivo* blood coagulation time in experimental groups following BMC transplantation.

Experimental group	Coagulation time 3 months post-transplantation (minutes)	Coagulation time 6 month post-transplantation minutes)
FVB/J [H2K^q]	3.7 ± 0.4	3.5 ± 0.3
C57BL/6J [H2K^b]	2.3 ± 0.4	2.4 ± 0.3
HA [H2K^b]	14.3 ± 1.17	14.2 ± 0.9
IR1	3.4 ± 0.3	3.5 ± 0.4
IR2	3.1 ± 0.3	-
IR3	3.0 ± 0.5	-
ST	2.6 ± 0.2	3.3 ± 0.7
IS	3.1 ± 0.4	-

6.4 Discussion

Management of genetic disorders pertaining to liver function is very cumbersome, expensive and frequently require life-long supplies of preventive medicine, as in Wilson Disease

Figure 6.12



Effect of increased plasma FVIII activity in phenotypic correction of hemophilia A
Experimental and control mice were subjected to tail cut challenge. The mice remaining alive after 24 hours were considered to have undergone phenotypic correction. Figures inside columns indicate the number of mice survived out of the total number of mice. Survival percentages other than 100% are given above the columns. **(A)** 3 months post-transplantation. **(B)** 6 months post transplantation. (IR1 to IR3) T_{reg} transplanted groups. (ST) Syngeneic transplantation group. (IS) Dexamethasone group. (HA) $FVIII^{-}/^{-}$. (WT) FVB/J. (B6) C57BL/6J

(Roberts and Schilsky, 2008) or the deficient protein, used in hemophilia A (Powell, 2000) and α1-antitrypsin deficiency (Ding *et al.*, 2011). Earlier in this study, it was shown that BM-derived hepatocytes can generate active coagulation FVIII in a FVIII deficient recipient, leading to correction of the disease condition. BMC transplantation could thus become a viable treatment option for hemophilia A if graft rejection can be prevented, as suggested by earlier findings in this study. To test this hypothesis, 3 different approaches were conceived to improve acceptability of FVB-GFP BMCs (MHC haplotype $H2K^q$) in the recipient HA mice (MHC haplotype $H2K^b$). In the first approach, a BM macrochimerism was generated in the recipient mice, which would be sufficient to induce tolerance to donor-specific cells (Ildstad and Sachs, 1984). However, the approach had to be abandoned, following very high mortality occurring within three months of allogeneic BM transplantation. The mice displayed symptoms typical of acute GVHD, such as weight loss, diarrhoea and skin abnormalities (Reddy and Ferrara, 2009).

The second approach in the present study was the use of dexamethasone to induce immune suppression. Dexamethasone inhibits lymphocyte proliferation through inhibition of inflammatory cytokines like IL-6, IL-10, TNF-α and IFN-γ (De *et al.*, 2002). It also induces FoxP3 expression in T cell cultures while preserving the suppressive capacity of T_{reg}s (Karagiannidis *et al.*, 2004; Dao *et al.*, 2004). HA mice undergoing dexamethasone treatment were liver damaged and were transplanted with uncommitted FVB-GFP BMCs. Although there was significant mortality in the group, enough mice survived for 3 months after transplantation to facilitate analysis for phenotypic correction of hemophilia.

The third approach was to induce donor-MHC specific tolerance in the recipient mice. It has been demonstrated that self-reactive human $CD4^+CD25^+$ T_{reg}s can be subverted into allopeptide-specific cells *in vitro* and be expanded to large cell numbers, and that similar *in vitro* expanded murine $CD4^+CD25^+$ T_{reg}s with indirect allospecificity are capable of inducing donor-specific experimental transplantation tolerance (Jiang *et al.*, 2006). In the present study, $CD4^+CD25^+$ T_{reg}s from HA mice were cultured in presence of FVB/J splenic dendritic cells in the presence of IL-2. This donor antigen-sensitized T_{reg}s were adoptively transferred to the recipient mice along with the BMCs. Allogeneic Lin^- BMC transplantation in the presence of T_{reg}s resulted in a survival rate comparable to that of syngeneic transplantation

model. This was in contrast with the observations from the other two groups where the survival rates were poor. While GVHD may have accounted for the high mortality rate among the BM-chimera, toxic effects of dexamethasone therapy may have been compounded by acetaminophen induced liver injury in the other group. Such toxicity factors however, are absent from immune tolerance induction approach. It has been demonstrated recently that donor antigen-stimulated recipient-type engineered T_{reg}s protects recipients from lethal GVHD in a murine model (Chen *et al.*, 2011). Taken together, these observations indicate that use of T_{reg}s is a safer and more efficient way of promoting allograft tolerance in a BMC transplant recipient.

Immunohistochemical analysis of recipient mouse livers 3 months after transplantation revealed the presence of donor derived hepatocytes in immunosuppressed as well as immunoregulated groups. Further, plasma FVIII analyses using ELISA and COATEST® revealed that the donor derived hepatocytes synthesized the full length active FVIII protein at amounts comparable to those found in syngeneic transplantation settings. These findings were further corroborated by the tests for *ex vivo* FVIII activity. The coagulation time of whole blood decreased significantly in the recipient mice of immunosuppressed and immunoregulated groups 3 months after transplantation, to levels that were comparable with both WT and syngeneic transplantation controls. In these quantitative tests, mice in IS group displayed consistently lower extent of phenotypic correction of hemophilia when compared to the IR groups and the ST group. However, this difference was statistically insignificant. Repetition of the quantitative tests for hemophilia A was carried out in an IR group and a ST group 6 months after transplantation. The IS group was not analyzed at this time point since high mortality rates had necessitated termination of the group at 3 months post-transplantation. The results of these tests showed that FVIII concentration, *in vitro* plasma FVIII activity and whole blood clotting time did not change significantly over the 3 month interval, suggesting that T_{reg} mediated immune tolerance induction is a sustainable mechanism to promote allograft survival.

Therapeutic correction in hemophilia A is possible even with low levels of FVIII in circulation. Transplantation of LSECs from WT mice to HA mice resulted in an increase in plasma FVIII activity to 14%-25% of normal which was above therapeutic level (Follenzi *et al.*, 2008). In

syngeneic transplantation settings, an increase in plasma FVIII activity to 30.6% of the normal was found to be sufficient to rescue mice from lethal hemorrhage (Yadav *et al.*, 2009). In the present study, the *in vitro* plasma FVIII activity among IS, IR and ST groups varied from 25%-44%. However, tail cut challenge experiments showed that these values are within therapeutic range. A repetition of tail cut challenge at 6 months post-transplantation time point once again demonstrated the correlation between the quantitative tests and survival of experimental mice. The high survival rates and consistent FVIII production makes immune tolerance induction using T_{reg}s the preferable method of enhancing phenotypic correction of hemophilia A under clinical transplantation settings.

Summary and Conclusions

The ability of bone marrow stem cells to differentiate in to hepatocytes both *in vitro* and *in vivo* has been well documented. This phenomenon brought in a new dimension in treatment of disorders of genetic origin, such as hemophilia A, which is due to a defective coagulation factor VIII. In a hemophilic individual the hepatocytes are unable to synthesize functional FVIII molecules, but this condition can be reversed by implanting functional hepatocytes in liver. Bone marrow transplantation provides a non-surgical option for accomplishing this task. Formation of anti-FVIII antibodies, otherwise known as inhibitors, is a known menace that jeopardizes the effectiveness of FVIII replacement therapy in humans. However, this does not seem to be the case in stem cell therapy. Presence of a tolerogenic environment in liver may be the definitive factor in preventing the formation of inhibitors against FVIII molecules synthesized by donor derived hepatocytes. However, it was to be seen that such immune tolerance could also result in acceptance of allogeneic bone marrow cells. The study of the efficiency of allotransplantation in achieving subsequent therapeutic correction of hemophilia A was of significant importance, since it is a question that will have to be answered during translation of these research findings into clinical medicine. Consequent to these investigations, it becomes imperative to devise some means of ensuring efficiency in stem cell therapy using allogeneic bone marrow cells to match that of stem cell therapy using syngeneic bone marrow cells.

The results presented in this study addresses each of these questions. Transplantation of MHC-matched undifferentiated bone marrow cells lead to generation of hepatocytes in recipient mouse liver. Transplantation of MHC-mismatched undifferentiated bone marrow cells however, was seen to result in very high mortality of recipient mice. This mortality points to the potential acute GVHD reaction which is a common occurrence after allogeneic hematopoietic stem cell transplantation in mice. The conditioning of the mice prior to transplantation by acetaminophen administration may have compounded the GVHD by promotion of inflammatory reactions. Predictably, no hepatocytes of allogeneic donor origin were located in recipient mouse liver.

Analysis of recipient mouse plasma revealed presence of donor derived FVIII protein in syngeneic recipient only. The FVIII present in plasma was found to be able to assist conversion of FX to FXa *in vitro*, which proved that it was functionally active. Further corroboration of this result was seen when freshly drawn peripheral blood was allowed to

coagulate. A marked reduction in coagulation time was observed in mice belonging to the syngeneic transplantation group, whereas mice in allogeneic transplantation group maintained a prolonged coagulation time, closely resembling that of the mutant control mice. When the recipient mice were subjected to surgical injury (tail clip) that was sufficiently serious to cause fatal hemorrhage in most of the mutant control mice, syngeneic recipients displayed the ability to survive by FVIII mediated hemostasis. When subjected to the same test, the allogeneic recipients did not survive. This was the final indication of the lack of effectiveness of allogeneic transplantation as a therapeutic option for phenotypic correction of hemophilia A.

Following this study, it was decided to compare the FVIII synthesis in hepatocytes and LSECs of donor origin in the recipient mouse liver. Immunohistochemical analysis showed that donor derived hepatocytes that expressed FVIII were present in WT and recipient mouse livers. FVIII protein was also visualized in LSECs of WT mouse. Further analysis by transmission electron microscopy revealed the presence of FVIII in both hepatocytes and LSECs in recipient mouse livers as was seen in WT mouse livers. RT-PCR and Real-Time RT-PCR analysis of highly purified hepatocyte and LSEC fractions showed that both fractions expressed *FVIII* light chain mRNA in comparable quantities. Hepatocytes expressed more *FVIII* gene after liver damage and reconstitution, whereas LSECs were found to be the major source of FVIII in WT mice. The cause of this differential expression is not understood and further research will be required to arrive at definite conclusions.

It became clear from the above study that immunomodulation will have to be achieved in the allogeneic recipient in order to prevent both graft rejection and mortality due to GVHD. As one of the approaches, bone marrow macrochimerism was induced in recipient mice. However, this led to near complete mortality necessitating the abandonment of the approach. A comparatively preferable approach would have been to establish mixed chimerism by infusion of T cell depleted donor and recipient bone marrow cells in to recipients, since mixed chimerism results in lower incidence and severity of GVHD. As another approach, recipient mice were subjected to an additional conditioning by administration of dexamethasone to induce non-specific immune suppression prior to allogeneic BMC transplantation. Although this approach resulted in high mortality rates, it also led to phenotypic changes similar to those observed in syngeneic recipients. The

recipient mouse plasma showed comparable rise in FVIII concentration in plasma. The increase of *in vitro* FVIII activity of allogeneic recipient plasma was also comparable with that of syngeneic recipient. The immunosuppressed mice further showed marked reduction in blood coagulation time and complete survival following surgical injury. These results showed that immunosuppression is a viable option for enhancing engraftment of allogeneic undifferentiated bone marrow cells in recipient mouse livers. However, the reason for the high mortality rate associated with this approach was not ascertained in this study. It is possible that administration of acetaminophen along with dexamethasone resulted in fatal liver injury. Assessment of this risk factor will require further studies using liver function tests with dose variations.

A third approach adopted for facilitating successful allotransplantation of bone marrow cells was the use of naturally occurring T_{reg} cells of the recipient origin. In order to establish donor-specific tolerance, recipient-derived T_{reg}s were sensitized *in vitro* towards donor antigens. Transplantation of sensitized T_{reg}s along with allogeneic undifferentiated bone marrow cells was found to result in much lower mortality rate when compared to the previously mentioned immunomodulatory approaches. Analysis of the recipient mice revealed phenotypic changes closely resembling those associated with syngeneic transplantation. The plasma FVIII concentration rose to comparable levels and did not significantly decrease over 6 months duration. Similar sustainable increase was seen in *in vitro* plasma FVIII activity. The reduction in blood coagulation time was comparable to syngeneic recipients and WT mice and was also maintained over 6 months. Complete survival was achieved after surgical trauma 3 months after transplantation. Proof of sustainable therapeutic correction was obtained 6 months after transplantation, when the mice subjected to surgical trauma showed complete survival. Taken together these results demonstrated that T_{reg}-mediated immune regulation effectively prevents rejection of allogeneic undifferentiated bone marrow cells by recipient mice, thereby facilitating their engraftment in injured liver and consequent differentiation into functional hepatocytes. This also indicates that T_{reg} may be adopted as a viable adjuvant option for enhancing allograft acceptance in humans.

In conclusion, the necessity to adopt a successful immunomodulatory approach to aid allogeneic stem cell therapy for hemophilia A was revealed by this study. It was shown that

both immune suppression and immune regulation could be applied successfully to this purpose. It was also shown that T_{reg} mediated immunoregulation is the safer method when compared to immune suppression using dexamethasone or induction of macrochimerism. However, further extensive research will be required to establish the optimum conditions regarding this approach, such as the dose of T_{reg}s required and the most desirable treatment regimen, before treatment of hemophilia A using T_{reg} aided allotransplantation of bone marrow progenitor cells becomes a reality.

Future directions

Transplantation immunology is indispensable study if research in stem cell biology has to be translated to provide favourable clinical outcomes. In the current study, stem cell transplantation was successfully employed for treatment of a fatal genetic disorder in mice, across histocompatibility barriers. It was shown that T_{reg}s of the recipient can be induced to protect the donor cells. However, many questions need to be answered before this work can have a significant impact in the clinical scenario.

1. The most obvious improvisation could be to find a more accessible source of T_{reg}s. It will be worthwhile to study the usefulness of peripheral blood T_{reg}s in this approach. T_{reg}s could be isolated from peripheral blood, expanded *in vitro* and used in the experiments. It will also be of importance to find out how long they could be maintained *in vitro*.
2. Treatment of hemophilia A required engraftment of the stem cells in liver followed by their proliferation. To facilitate this, the cells were delivered through systemic circulation. It consequently resulted in high dilution of cells. It may be possible that in case of another disease of liver *e.g.*, Wilson disease, the cells could be delivered through hepatic portal system or spleen, which would mean that much fewer cells are enough to ensure clinical success. Therefore, the approach of bone marrow progenitor cell therapy in conjunction with T_{reg} transplantation may be adapted to treat other genetic disorders with greater efficiency.
3. The uncommitted cells of the bone marrow were used in this study as progenitors of neo-hepatocytes. However, the exact phenotype of the cells that undergo engraftment and differentiation has not been studied. It is not known if T_{reg} mediated immune regulation protects every cell type transplanted. Knowledge of the phenotype of the stem cell population most efficient in repopulating the recipient liver may help reduces the antigenic load during transplantation. This could itself have major effects in the magnitude of inflammatory and immune responses following transplantation, long term survival of the graft, and hence in the well being of the patient.

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Publications

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