Immune-mediated hepatitis drives low-level fusion between hepatocytes and adult bone marrow cells

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Background/Aims: The role of adult bone marrow-derived cells (BMC) in hepatic regeneration is controversial. Both transdifferentiation of BMC as well as fusion with hepatocytes have been suggested in toxin-based and genetic selection models.

Methods: We have developed a transgenic mouse model of immune-mediated hepatitis to clarify the role of BMC in liver regeneration following injury mediated by T cells.

Results: Repeated adoptive transfer of transgenic T cells into bone marrow chimeras resulted in multiple waves of hepatitis. Hepatocytes derived from donor bone marrow were identified using a self-protein that does not interfere with hepatocyte function and proliferation in recipient animals. Some cells contained one recipient nucleus and another independent donor bone marrow-derived nucleus, suggesting that cellular fusion plays some role in liver repair after immune hepatitis. However, despite pronounced infiltration by myeloid cells, the frequency of fusion was extremely low.

Conclusions: This study provides a unique, clinically relevant model in which fusion hepatocytes can be purified and characterized by the expression of donor MHC antigen. It demonstrates that although fusion between BMC and hepatocytes occurs under conditions of inflammation that correspond to human disease, its frequency needs to be increased to be of any therapeutic value.

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1. Introduction

Several recent studies have shown that cells derived from adult bone marrow can contribute to liver regeneration following hepatic injury [1–8]. Initial investigations that

combined inhibition of mature hepatocyte proliferation with liver injury identified bone marrow cells as the source of liver derived endogenous oval cells in mouse models [1]. The most widely cited model used fumarylacetoacetate hydrolase (Fah) knock out mice [2]. Fah$^{-/-}$ mice die from progressive liver failure if not treated with an appropriate diet. In livers of Fah$^{-/-}$ mice treated with highly purified hematopoietic stem cells from wild type donor mice, up to 50% of hepatocytes expressed a donor marrow derived phenotype that resulted in maintenance of normal liver function. Subsequent studies presented evidence that liver

regeneration in the Fah$^{-/-}$ model occurred by cellular fusion between donor marrow cells and hepatocytes [8]. This was confirmed using an in vivo Cre/Lox recombination method in similar regeneration models, further demonstrating fusion of bone marrow cells with hepatocytes [6]. Parallel studies have additionally demonstrated that fusion of donor marrow cells with existing organ cells occurs in many but not all model systems [3–5,7]. For hepatocytes, fusion is dependent predominantly on cells from the myeloid lineage rather than hematopietic stem cells [9,10].

It is clear from these studies that bone marrow cells have the ability to repopulate injured livers. These observations are extremely valuable as they offer both new opportunities to understand the nature of developmental cell differentiation and the identification of potential therapeutic opportunities. However, based on the evidence presented so far, it remains unknown whether this phenomenon has any clinical relevance [11,12]. All models presented recently induced liver damage by either non-physiologic hepatotoxic agents or by genetic pressure models, both of which represent highly artificial research models that mimic few clinical liver diseases. Furthermore, previous models did not allow sorting and purification of bone marrow-derived hepatocytes.

In the current studies, we utilized a transgenic mouse model of immune-mediated hepatitis to study bone marrow cell plasticity in a situation in which recurrent inflammation creates a milieu that is comparable to human liver pathophysiology [13] and in which hepatocytes expressing bone marrow-derived markers could be directly sorted by flow cytometry. Our findings indicate that some bone marrow derived cells homed to the injured liver and fused with resident hepatocytes. Cellular fusion was, however, rare and its frequency needs to be further increased to be of any potential therapeutic value.

2. Methods

2.1. Mice

T cell receptor (TCR) and MHC (H-2K^b) haplotype transgenic mice on a B10.BR background (H-2K^k) have been described elsewhere [13,14]. Des-TCR mice express a transgenic TCR specific for H-2K^b and a self-peptide on all T cells (DES mice). 178.3 mice, on a B10.BR background, ubiquitously express the H-2K^b transgene under the control of its own promoter. Mice were bred and cared for in the Centenary Institute animal house.

2.2. Generation of bone marrow chimeras and induction of hepatitis

Female B10.BR (H-2K^k) recipient mice were pretreated twice with the hepatocyte selective mitotic-inhibitor Retrorsine (Sigma-Aldrich, Germany, #R0382) 7 days apart [15]. Mice were then subjected to total body irradiation with 6.5 Gy 6 h before transplantation of 50×10^6 total bone marrow cells (BMC) isolated from either 178.3 or B10.BR male mice. Six weeks after bone marrow transplantation, recipient animals were injected intravenously with 15×10^6 syngeneic lymph node T cells from female Des-TCR mice. This induced a severe but non-lethal, immune

mediated bystander hepatitis as previously described [13]. The same recipient mice were then sequentially injected four times at intervals of 1–2 weeks with the same number of Des-TCR T cells to induce successive waves of hepatitis. One group of animals was additionally subjected to two 5-day courses (hepatitis cycles 1 and 4) of recombinant human GCSF at a dose of 1 mg/kg s.c. [16]. One week after the last T cell injection, animals were harvested and livers were perfused as described below. The initial group size of eight mice decreased due to non antigen-specific death of some mice during the course of the complex treatment (deaths due to irradiation, embolism following injections, etc.).

2.3. Liver perfusion and hepatocyte purification

To obtain pure single cell suspensions of hepatocytes, abdomens of euthanised animals were opened via a midline incision and the inferior vena cava was canulated with a 26 gauge i.v. canula connected to a peristaltic pump. Livers were retrogradely perfused with approximately 25 ml of HBSS medium (Gibco, Invitrogen, Australia) at 37 °C. The liver was again perfused with 25 ml of HBSS medium containing 0.5 mmol EDTA which was then washed out by perfusing with another 25 ml of plain HBSS medium followed by perfusion with the same amount of HBSS medium containing 5 mmol calcium hydrochloride and 0.5 mg/ml hepatocyte purification grade collagenase (Sigma-Aldrich, Australia). The resulting cell suspension was spun at 500 rpm/50 g for 3 min at 4 °C and washed in RPMI three times and then once again using a PBS wash. Washes were followed by gradient centrifugation with 35% isotonic Percoll (Amersham Biosciences, Australia) at 2000 rpm for 10 min at 20 °C to select for viable single cells.

2.4. Immunohistochemistry (IHC)

Immunostaining was performed according to standard protocols using commercially available antibodies against murine H-2K^b, H-2K^k, CD45, CD8, CD4, Mac-1, Gr-1, (all from BD Pharmingen, La Jolla, CA) and pan-cytokeratin (Sigma-Aldrich, Australia) in combination with appropriate fluorochromes or peroxidase conjugated anti-Ig secondary antibodies or streptavidin conjugates (BD Pharmingen or Roche, Germany). H-2K^b staining was achieved using H-2K^b antibodies directly coupled to FITC and anti-FITC secondary antibodies (Roche Germany). Xenogenic goat or swine serum and isogenic serum were used to decrease nonspecific staining. DAB substrate (Sigma-Aldrich, Australia) was used in IHC for colour development. Slides were counterstained with hematoxylin and Scotts Blue solution and mounted with DPX (Sigma-Aldrich, Australia #44581).

2.5. Fluorescence activated cell sorting (FACS) and immunofluorescence staining

FACS analyses were carried out on a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA) and data analysed with FlowJo software (Tree Star Inc., USA). Experimental animals were always directly compared to controls of the same set of experiments. Purified hepatocytes were stained with biotinylated anti-H-2K^b antibody (BD Pharmingen) and streptavidin-Alexa 594 (Molecular Probes, Australia). Cells were then gently macro-sorted using a FACSVantage (BD Biosciences, Australia) for the H-2K^b positive, PI negative cell population using a 200 μm nozzle. Purity of the sorts was greater than 95%, although the yield of living cells after the sort was reduced to approximately 50%. Cytospin preparations were stained with either anti-K^b, anti-CD45.2 or anti-pan-cytokeratin antibodies coupled to FITC for 1 h at 4 °C. Fluorescent nuclear counterstain was achieved using DAPI (Sigma-Aldrich).

2.6. Fluorescence in situ hybridization

Cytospins were fixed for 10 min in ice cold 1:1 methanol–acetone at −20 °C and rinsed in 2× saline–sodium citrate buffer (SSC) for 5 min each. Slides were then immersed in 60% formamide in 2×SSC at 70 °C for 3 min and then immediately dehydrated through passage in ice-cold 70, 95 and 100% ethanol. X chromosome probe and DOP-PCR murine Y paint probe (generously donated by Dr Diane Krause, Yale University, CT, USA)
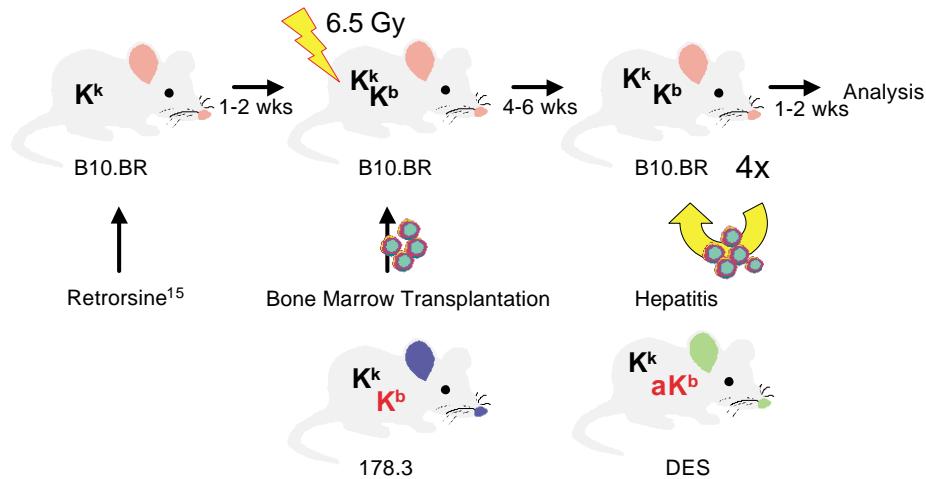
Fig. 1. Treatment scheme. Retrorsine Pretreated B10.BR recipients were sublethally irradiated and transplanted with 178.3 donor bone marrow as indicated. After stable establishment of peripheral macrochimerism, recipients were subjected to serial lymph node cell injections to induce hepatitis. Livers were analysed 1–2 weeks after the last T cell injection.

were digoxin and biotin labeled, respectively (DIG-Nick Translation Mix, Roche, Penzberg, Germany; BioNick Labeling System, Invitrogen, Carlsbad, CA, USA). Labeled probes were denatured in LSI/WCP Hybridization Buffer (Vysis, Downers Grove, IL, USA) in the presence of Cot-1 DNA for 7 min at 75 °C and re-annealed at 37 °C for at least 1 h [17]. Briefly, 5 μl of probe mix was applied to each slide and covered with a 12 mm-diameter coverslip. Slides were incubated overnight at 37 °C in a humidified chamber. After removal of the coverslips, slides were washed with 50% formamide in $2\times$SSC for 2 min at 42 °C and rinsed in $2\times$SSC. Slides were then incubated for 1 h at RT with blocking buffer (4% BSA, 0.2% Tween-20 in $2\times$SSC). Cy3-conjugated mouse anti-digoxin (Jackson Immunoresearch, West Grove, PA, USA) and fluorescein-conjugated streptavidin (Jackson Immunoresearch) were diluted in the above blocking buffer at 1:50 ratio each, and applied to the slides. After a 2 h incubation at RT, slides were washed for 10 min with 0.1% Tween 20 in $4\times$SSC. Slides were then washed three times for 5 min in 10 mM PBS, rinsed in ddH2O and mounted in Aquamount (Polysciences, Warrington, PA, USA). Images were captured by an Olympus BX50 confocal microscope equipped with a krypton–argon mixed gas multiline laser and analysed with BioRad Lasersharp 2000 software.

3. Results

3.1. Induction of hepatitis in B10.BR bone marrow recipients

Immune-mediated hepatitis was induced in chimeric female B10.BR mice by intravenous injection of lymph node cells from syngeneic Des-TCR female donors. Previous studies have shown that chimeric animals injected with a single injection of Des-TCR T cells developed a transient hepatitis peaking at day 2. T cells were activated in an antigen-specific manner by bone marrow derived APCs in the liver and subsequently caused bystander destruction of non-antigen bearing hepatocytes [13]. Although subsequent peripheral deletion of donor T cells was observed in this model, injection of recipient mice with a second cohort of Des-TCR lymph node cells resulted in a second wave of transient hepatitis of similar intensity (Bowen, unpublished observation). To induce more persistent liver damage and to favour hepatocyte regeneration in the current study, recipient

mice were injected with four cohorts of lymph node-derived Des-TCR cells. Injections were administered at intervals of 1–2 weeks (Fig. 1). This protocol resulted in four equal waves of hepatitis as suggested by comparable average ALT levels after the first and fourth injection (Fig. 2). Histologically there was evidence of portal and scattered lobular inflammation in association with occasional Councilman bodies representing apopotoic hepatocytes. The histological picture

Mean	12.0	10.2	87.6	69.7
SEM	2.90	5.17	21.53	23.05
Injection	1st	4th	1st	4th
	Control		Hepatitis	
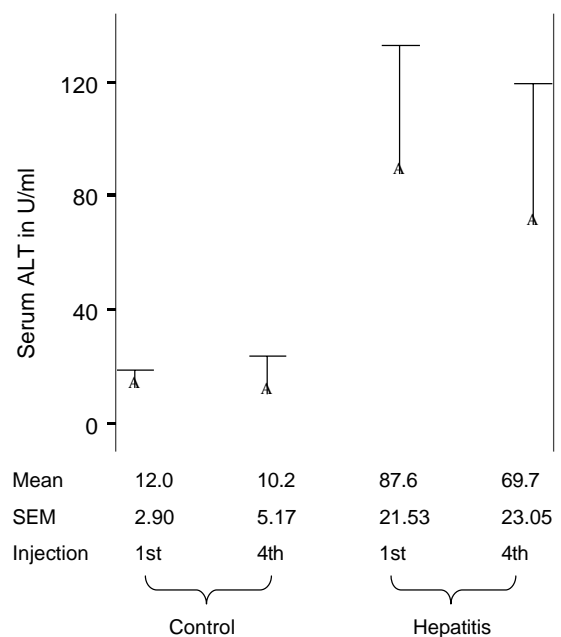
Fig. 2. Mean serum ALT levels after the first and fourth injection of transgenic T cells. Serum ALT levels were analysed after the first and the fourth injection of transgenic T cells in animals transplanted with syngenic bone marrow (group #1) versus animals transplanted with transgenic bone marrow (groups #2 and #3). Only animals receiving H-2K^b positive bone marrow from 178.3 mice developed hepatitis with increased ALT levels. Error bars show 95% confidence intervals. SEM: standard error of the mean.
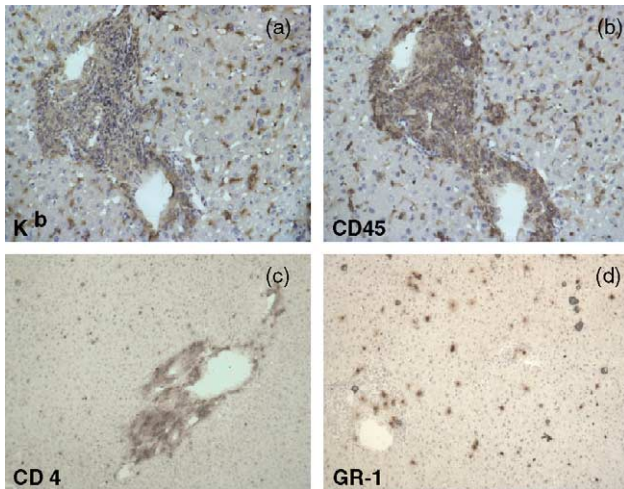
Fig. 3. Immunohistochemistry of livers after four courses of immune-mediated hepatitis. Livers were harvested from animals of group #2 developing hepatitis, snap frozen and stained by immunohistochemistry. (a) Representative liver showing infiltration of H-2K^b positive mononuclear cells from the donor bone marrow (brown pigmentation following staining with anti-H-2K^b-FITC mAb and anti-FITC-Fab fragments coupled to peroxidase). The majority of donor cells were CD45 positive, representing the ongoing inflammatory response by hematopoietic cells (b). CD4 positive T cells (c) were mainly observed within the portal field, whereas Gr-1 positive monomyelocytic cells displayed a more parenchymal distribution (d).

characteristic of ongoing inflammatory destruction in mice grafted with 178.3 bone marrow (Fig. 3). In contrast, damage and increased ALT levels were not observed in control animals subjected to isogenic bone marrow transplantation followed by the same T cell injections. Most cells infiltrating the liver of chimeric mice expressed H-2K^b indicating that they were of donor marrow origin (Fig. 3(a)). Serial staining demonstrated that most of the invading cells were CD45 positive leukocytes (Fig. 3(b)). These leukocytes included CD4 and CD8 T cells, but also macrophages and granulocytes as assessed by expression of Mac-1 or Gr-1, respectively, (Fig. 3(c) and (d) and data not shown).

3.2. Flow cytometric analysis of single liver cell suspensions demonstrated that some hepatocytes expressed the H-2K^b bone marrow-derived marker

To determine whether successive Des-TCR T cell injections could increase liver regeneration from the bone marrow in our present model, hepatocytes were isolated and analysed by flow cytometry for H-2K^b expression. Hepatocytes were several times larger then lymphocytes and could easily be distinguished by their side and forward scatter profile (Fig. 4(a)). Hepatocytes from unmanipulated B10.BR and H-2K^b-expressing 178.3 transgenic mice were used as controls (Fig. 4(b) or (c)). In 178.3 bone marrow recipients serially injected with transgenic T cells, H-2K^b positive cells could be detected within the hepatocyte size gate (compare Fig. 4(d) (control) to Fig. 4(e)) (animal from group #3, upper right quadrants). The percentage of
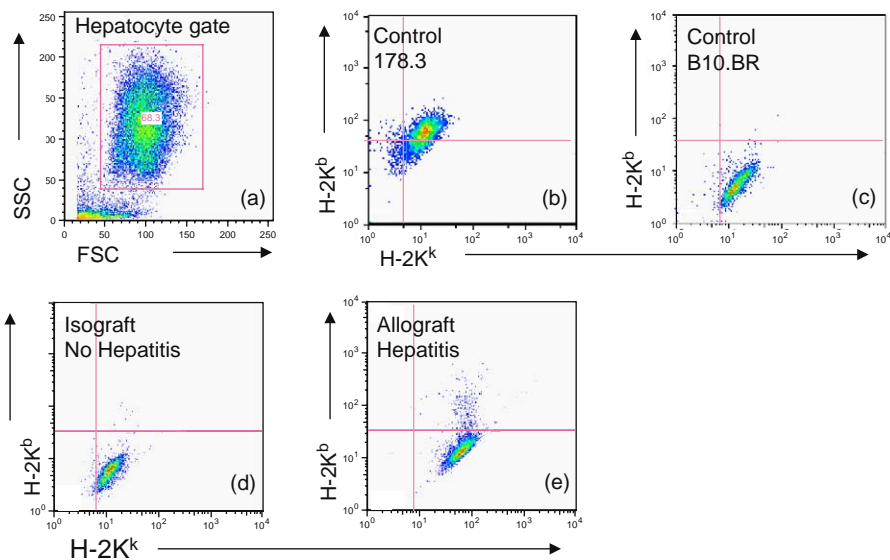
was, therefore, very similar to mild acute viral hepatitis in humans with similar ALT levels.

Two weeks after the last T cell injection, gross macroscopic changes were observed in the liver parenchyma

Fig. 4. FACS analysis of purified hepatocytic populations. Hepatocytes were purified from livers of treated animals as described above. These hepatocytes formed a homogenous population clearly distinct from leukocytes when assessed by FACS (a). Control 178.3 (b) and B10.BR (c) hepatocytes stained with the appropriate MHC markers with highest specificity (anti-H-2K^b in combination with anti-H-2K^k). Comparing similar stains from B10.BR-chimeric (d) and 178.3-chimeric mice (e—from group#3) revealed the presence of hepatocytes expressing the donor derived MHC antigen (H-2K^b) in animals from the hepatitis groups. These cells were strictly PI negative viable cells (not shown) and were included in a size gate appropriate for hepatocytes (a).

Table 1
Treatment groups

Group	N	BM Recipient	BM Donor	Retror-sine[15]	GCSF	% Hepatocyte chimerism	Mean ± STD	p vs #1
#1	6	B10.BR (f)	B10.BR (m)	No	No	0.09, 0.23, 0.29, 0.82, 0.83, 0.98	0.54 ± 0.37	–
#2	7	B10.BR (f)	178.3 (m)	No	No	0.27, 0.31, 0.81, 1.32, 1.77, 2.31, 3.01	1.40 ± 1.03	0.075
#3	4	B10.BR (f)	178.3 (m)	Yes	No	0.88, 1.21, 4.64, 6.92	3.41 ± 2.89	0.141
#4	1	B10.BR (f)	178.3 (m)	Yes	Yes	0.88, 1.50, 3.03, 5.43	2.71 ± 2.02	0.120

Female B10.BR mice were engrafted with either male B10.BR control bone marrow (group #1) or bone marrow from H-2K^b transgenic male 178.3 donors (groups #2–#4). Hepatitis was induced by injection of transgenic Des-TCR T cells. Animals in groups #3 and #4 were additionally treated with Retrorsine or GCSF as indicated. Chimerism of low speed gradient centrifugation purified hepatocytic cell populations was assessed by flow cytometric analysis.

hepatocytes, however, expressing donor bone marrow derived antigen was low (1.40% ± 1.03% versus 0.54 ± 0.37, $P = 0.07$ (independent sample t-test), Table 1). Most H-2K^b positive cells did not express CD45, suggesting that leukocyte contamination and adhesion-artefacts were rare. The upregulation of MHC molecule expression by all hepatocytes isolated from animals developing hepatitis (Fig. 4(e)) probably occurred as a result of inflammation.

Our results indicate that despite severe liver damage and the presence of high numbers of myelomonocytic cells, the percentage of H2-K^b positive hepatocytes was low and at the edge of statistical significance in comparison to controls. This percentage was slightly increased following treatment of recipient mice with Retrorsine alone or in combination with GCSF (Table 1). Thus, even under conditions that inhibited endogenous hepatocyte proliferation and/or increased mobilization of BMCs, bone marrow-derived hepatocytes were extremely rare events.

As the percentage of H-2K^b hepatocytes was low applying flow methods, we investigated further whether H-2K^b positive events included hepatocytes that genuinely express H-2K^b. In these further investigations, single cells from hepatitis animals with hepatocyte morphology (Fig. 5(a)) were identified which stained positive for both MHC haplotypes by fluorescence microscopy (Fig. 5(d)), suggesting that they genuinely expressed H-2K^b. Under the same conditions, no H-2K^b staining was observed in hepatocytes isolated from control mice (Fig. 5(b) or (c)). To confirm these results cell suspensions isolated from 178.3 chimeras were stained with anti-H2-K^b mAb, sorted by FACS and subsequently analysed by fluorescence microscopy. Most of these sorted cells coexpressed cytokeratin, a marker not typically expressed by hematopoietic cells from the bone marrow (Fig. 5(e)). Most H-2K^{b+} cells had a hepatocyte morphology and were clearly CD45$^-$ (Fig. 5(f), cells 2 and 3). However, a fraction of sorted H-2K^{b+} cells were CD45$^+$, indicating that they were either hepatocytes still expressing CD45 after fusion, hematopoietic cells on their way to differentiate into hepatocytes or large reactive leukocytes (Fig. 5(f), cell 1). Collectively, these experiments suggest that although their frequency was very low, some hepatocytes genuinely expressed the donor bone marrow-derived MHC antigen H-2K^b.

3.3. Evidence for fusion between donor bone marrow derived cells and recipient hepatocytes in the hepatitis model.

Sorted H-2K^{b+} hepatocytes were further analysed for their X and Y chromosome content using the FISH
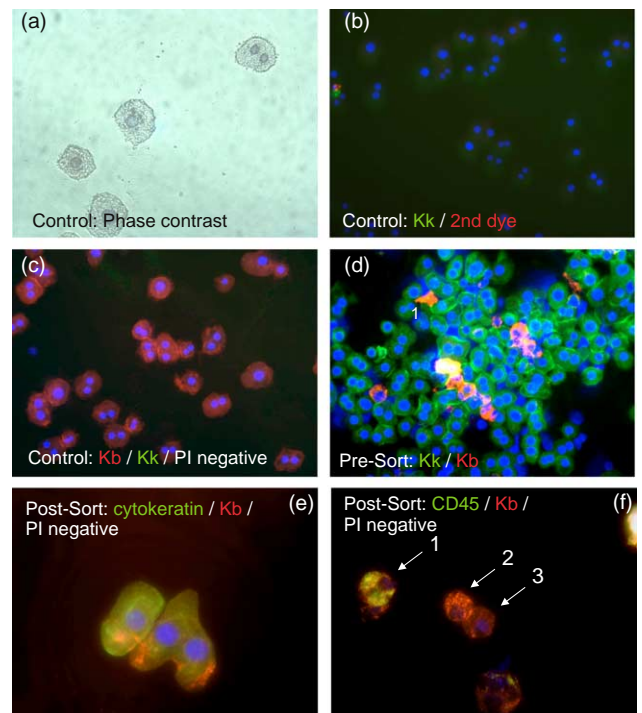
Fig. 5. Immunofluorescence analysis of non-sorted and sorted hepatocytes. Purified hepatocytes were sorted by FACS and then assessed by immunofluorescence staining. All cells preparations were initially examined by phase contrast microscopy to check for hepatocyte morphology and exclude cell doublets (a). Control C57BL/6 PI$^-$ hepatocytes were stained with anti-K^k-FITC (green) and Avidin-Alexa594 (red) to exclude non-specific binding of the secondary mAb (b), or costained with anti-H-2K^k-FITC (green) and anti-H-2K^b + Avidin-Alexa594 (red) after sorting for PI only to ensure specific staining of viable cells (c). Most cells of hepatitis animals had hepatocyte morphology, expressed H-2K^k but only a few expressed H-2K^b pre-sorting (d). PI$^-$ cells binding anti-H-2K^b and Avidin-Alexa594 (red) were sorted by flow cytometry and costained with anti-cytokeratin-FITC (e) or anti-CD45-FITC (f). Although some H-2K^{b+} cells costained with CD45, indicating that they were either large leukocytes or hepatocytic cells still expressing CD45 (cell# 1 in f), most H-2K^{b+} cells of hepatocyte morphology were CD45$^-$ and cytokeratin$^+$ (cells #2 and 3 in f).

technique. Normal hepatocytes from male control animals displayed one to four nuclei (in general two) with clear signals for X and Y (Fig. 6(a)—XYXY in this cell). Fifteen thousand H-2K^b positive cells with hepatocyte morphology, obtained from livers of chimeric female mice in which hepatitis was induced, were analysed after FACS sorting. Out of these cells, fifteen contained nuclei with heterogeneous X–Y content (1 in 10^3). In most cases, only one nucleus of the 2–4 included a Y chromosome (Fig. 6(b) and (c), XXXY and XXXXXY, respectively), suggesting that these cells were derived from fusion between a donor hematopoietic male cell and a recipient female hepatocyte. Contaminating leukocytes contained a single male nucleus only. The frequency of fusion events in the current model can, therefore, be calculated to be 1 in 10^5 of total hepatocytes ($1–10^3$ times $1–10^2$ ($\sim$1%) enrichment in the sorting process for H2-K^b).

4. Discussion

There is growing evidence that bone marrow (stem) cells can contribute to non-hematopoietic tissue development or regeneration after injury [1–3,6,18–21]. Most studies to date analyzing the liver have utilized rodent systems with genetic deficiencies or have applied toxins in their regeneration protocols [2,7,8]. This paper investigates for the first time the bone marrow to hepatocyte cell plasticity in a setting of immune-mediated hepatitis [13,22]. Clinical hepatitis, induced by hepatotrophic viruses or autoimmune disorders, is a common pathology of human liver with millions of per annum cases worldwide [23,24]. Whether bone marrow cell plasticity plays a significant role in physiological turnover or in human disease and whether it can be exploited for the purposes of regenerative medicine remains unclear [11,12, 25]. Using Fah knock-out mice, in which de novo generated hepatocytes have a selection advantage, hepatocyte macro-chimerism can occur after hematopoietc stem cell transplantation resulting in correction of the underlying metabolic disorder [2]. The initial assumption that bone marrow stem cells transdifferentiated into donor-derived hepatocytes was, however, amended in a later report showing that bone marrow-derived cells predominantly fuse with genetically deficient Fah$^{-/-}$ hepatocytes [7,8]. Using the same model, similar results have been achieved by transplanting only single hematopoietic stem cells [3,9]. It has been demonstrated that cells of the myelo-monocytic lineage, including fully differentiated macrophages, were capable of fusion [3,9,26]. Cells of myelo-monocytic origin may be the privileged fusion partners for hepatocytes, because they already possess the machinery allowing them to fuse with other cells [27,28]. Cellular fusion of hepatocytes and bone marrow-derived cells and/or macrophages, therefore, seems to be the predominant mechanism of plasticity in liver [10], although it is extremely difficult to formally rule out bone marrow cell transdifferentiation (or
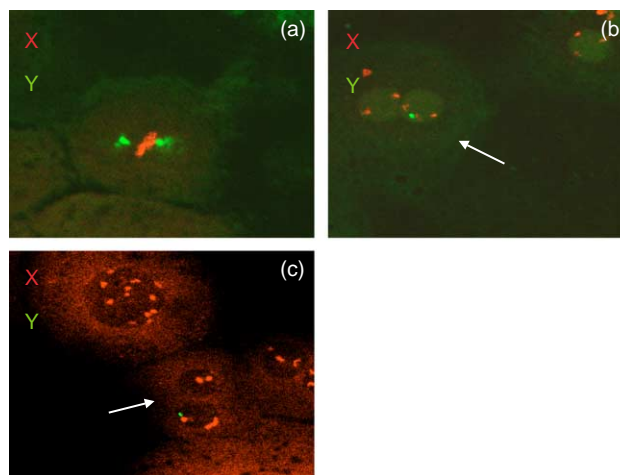
Fig. 6. FISH analysis on hepatocytes sorted for the donor derived H-2 K^b antigen. Cytospin preparations of hepatocytes purified from the liver of male C57BL/6 mice (a) or H-2K^{b+} FACS-sorted hepatocytes from female 178.3 chimeric mice developing hepatitis (b and c) were co-hybridized with biotinylated male Y and digoxin-conjugated female X probes in combination with avidin-FITC (green) and anti-digoxin-PE (red). Most male control hepatocytes showed two independent nuclei each containing at least one X and Y chromosome (a). Some hepatocytes sorted from 178.3 female chimeras contained one or more female nuclei containing at least one set of X chromosomes in addition to independent male nuclei from the bone marrow donor with at least one X and Y chromosome each in the majority of cases (b and c).

even transdifferentiation before fusion) as reported in some other studies [20,25,29].

Our model fulfilled two hypothetical criteria, which can be considered to be prerequisites for bone marrow cell plasticity—a state of regeneration upon injury and presence of specialized bone marrow derived leukocytes in the regenerative environment. As the frequency of BMC to hepatocyte cell fusion was anticipated to be low, we have combined flow analysis with single cell staining and cytogenetics to screen for fusion cells. Thus, we could show that despite severe liver damage and high numbers of infiltrating leukocytes (including Mac-1 and GR-1 positive cells of myelomonocytic origin), fusion is a rare event that occurs only within a very small fraction of the hepatocyte population (estimated frequency 1 in 5×10^4–1 in 5×10^5). Fusion hepatocytes were purified by flow sorting and further analysed. It cannot be formally ruled out that during the purification process fusion hepatocytes reacted differently to the isolation process whereby their number would have been under- or overestimated. However, this must be considered unlikely, as their physical properties should be unchanged as compared to normal hepatocytes. Furthermore, we cannot exclude that Des-TCR T cells preferentially killed de novo generated H-2K^{b+} hepatocytes, We think that this is unlikely as immune responses to liver antigens are known to be associated with tolerance rather than rejection [22]. We, therefore, expect that T cells accumulating in the liver will become tolerant to self-antigen following the initial bystander hepatitis and would not further destroy newly

generated hepatocytes expressing H-2K^b following fusion with bone marrow cells.

When 178.3 chimeras were pre-treated with the mitoinhibitory substance Retrorsine, which inhibits mature hepatocyte proliferation [15,30] or were exposed to GCSF to mobilize bone marrow (stem) cells, the number of fusion events was not significantly increased. The occurrence of fusion events in our model corresponds to the fusion frequency in the Fah$^{-/-}$ mouse if selection pressure is not applied [31]. When NTBC is discontinued in the Fah model, the frequency of fusion has been shown to increase significantly probably due to the constant selection pressure imposed on hepatocytes. This conclusion is also consistent with the finding that more than 30% hepatocytes that stained positive for the Y chromosome were found in a human male suffering from recurrent hepatitis C, after having received a female liver graft [20]. That genetic selection pressure can drive the generation of donor derived hepatocytes after bone marrow transplantation was additionally outlined in a transgenic mouse model expressing Bcl-2 under a liver specific promoter. Injection of anti-Fas antibodies into bone marrow chimeras selectively amplified donor derived hepatocytes in this model to levels which could be of therapeutic value [32].

In conclusion, we have developed a novel transgenic mouse transplantation model, which enables the purification and identification of fusion hepatocytes by a non-selective MHC antigen. This model is a valuable tool for further molecular analysis of fusion cells in the future. In addition it mimics some of the severe conditions that would occur during common human disease and, therefore, complements the Fah$^{-/-}$ model and other models. We show that despite severe liver damage resulting in abundant leukocyte infiltration, fusion of BMC with hepatocytes and subsequent expression of a bone marrow-derived MHC antigen in the liver is a rare event. Although clearly subtherapeutic at this stage, treatments aimed to increase the frequency of fusion events could represent a potential strategy for integration of exogenous genes by future cell transplant strategies.

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