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Ectopic expression of murine CD47 minimizes macrophage
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Title page**HEP-11-2245****Ectopic expression of murine CD47 minimizes macrophage rejection of human hepatocyte xenografts in immunodeficient mice**

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Footnote page

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List of abbreviations:

GFP: enhanced green fluorescence protein, Cd47: murine CD47, hCD47: human CD47, SIRP α : Signal inhibitory regulatory protein α , uPA: urokinase-type plasminogen activator, MOI: multiplicity of infection, hALB: human Albumin.

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Abstract

Macrophages play an important role in the rejection of xenogeneic cells and therefore represent a major obstacle to generating chimeric mice with human xenografts that are useful tools for basic and preclinical medical research. The inhibitory SIRP α receptor is a negative regulator of macrophage phagocytic activity and interacts in a species-specific fashion with its ligand CD47. Furthermore, SIRP α polymorphism amongst laboratory mouse strains significantly affects the extent of human CD47-mediated toleration of human xenotransplants. Aiming to minimize macrophage activity and thus optimize human cell engraftment in immunodeficient mice, we lentivirally transduced murine CD47 (Cd47) into human liver cells.

Human HepG2 liver cells expressing Cd47 were less frequently contacted and phagocytosed by murine RAW264.7 macrophages *in vitro* than their Cd47-negative counterparts. For the generation of human-mouse chimeric livers in immunodeficient BALB- Δ RAG/ γ_c -uPA mice, freshly thawed cryopreserved human hepatocytes were transduced with a lentiviral expression vector for Cd47 using a refined *in vitro* transduction protocol immediately before transplantation. *In vivo*, Cd47-positive human primary hepatocytes were selectively retained following engraftment in immunodeficient mice, leading to at least a doubling of liver repopulation efficiencies.

Conclusion: We conclude that ectopic expression of murine Cd47 in human hepatocytes selectively favors engraftment upon transplantation into mice, a finding that should have a profound impact on the generation of robust humanized small animal models. Moreover, dominance of ectopically expressed murine Cd47 over endogenous human CD47 should also widen the spectrum of immunodeficient mouse strains suitable for humanization.

Introduction

The interest in the development and use of humanized mice to investigate human biological systems *in vivo* outside the human body is continuously growing. Humanized mice are either transgenic animals expressing human genes and/or immunodeficient mice engrafted with human cells or tissues (1). Immunodeficient mice repopulated with human hepatocytes have already proven useful for the study of hepatitis virus life cycles and new antiviral approaches (2, 3) and various immunodeficient strains have emerged from different labs over time (4).

In this study we used a mouse model for the generation of human-mouse chimeric livers based on Rag2^{-/-}IL-2Rγ^{null} mice with BALB/c background that are homozygously transgenic for albumin-promoter-enhancer driven urokinase-type plasminogen activator expression (Alb-uPA^{tg+/+}) (5-9). This BALB-ΔRAG/γ_c-uPA mouse model is characterized by severe damage of the liver architecture, requiring transplantation of uPA-negative hepatocytes within 2-5 weeks after birth for survival. Transplanted human cells integrate well into the murine liver parenchyma and replace endogenous hepatocytes (10).

Although most of the immune system is dysfunctional in this model, macrophages remain intact (11) and are mainly responsible for suboptimal efficiencies of xenotransplantation. It has been shown that depletion of macrophages upon clodronate treatment supporting hepatocyte xenotransplantation improves engraftment levels (7).

For many applications, optimal engraftment and repopulation are critical. For example, a minimal repopulation threshold has to be reached to investigate the replication of human hepatotropic pathogens (*e.g.* HBV, HCV, malaria parasite) in chimeric mouse livers (7, 12, 13).

The transmembrane protein CD47 plays a key role in immune regulation of macrophage rejection. It was shown that CD47-deficient red blood cells injected into wild type mice were rapidly eliminated by spleen macrophages, whereas CD47⁺ erythrocytes were not (14). Signal

regulatory inhibitory protein α (SIRP α) has been identified as a binding partner of CD47 (15). SIRP α -deficient peritoneal macrophages eliminated CD47⁺ red blood cells, indicating an interaction between CD47 and SIRP α for specific cell recognition of “self” and inactivation of macrophages (16). Upon species-specific binding of CD47 to SIRP α a signal cascade is triggered which ultimately leads to macrophage inactivation, preventing phagocytosis (17). Phagocytic assays revealed that a porcine B-lymphoma-like cell line (LCL) transfected with human CD47 had a better survival than wild type-LCL when incubated with human macrophages *in vitro* (18). Furthermore, Legrand *et al.* could demonstrate that ectopic Cd47-expression in transplanted human hematopoietic cells is required for optimal human T- and natural killer-cell homeostasis in mice (19).

Here we show that stable ectopic expression of recombinant murine CD47 (Cd47) in human liver cells significantly reduces mouse macrophage activity *in vitro* and that the introduction of Cd47 into primary human hepatocytes confers a positive selective advantage upon engraftment into the mouse liver *in vivo*.

Materials and Methods

Cell culture. Human HepG2 cells and the fibrosarcoma cell line HT1080 were cultured in DMEM supplemented with 10% FCS (PAA), 200 U penicillin/streptomycin, 1 mM L-glutamine (PAA) in 5% CO₂. Cryopreserved primary human hepatocytes (BD Biosciences) were cultured for FACS analysis (FACS Calibur, BD) in hepatocyte culture medium (HCM, Lonza).

Cloning of murine Cd47. Cd47 was amplified using the Cd47 cloning primers (Supplementary Material 1) from mouse liver cDNA and subcloned for sequencing. Sequencing of BALB/c mouse liver amplicons revealed one base exchange at position 716 of the coding sequence compared to the published AK151922 mRNA of Cd47, resulting in the amino acid sequence change I213V. This point mutation was confirmed in cDNAs from C57BL/6 mice and the Hepa1-6 tumor cell line. The modified version of AK151922 has been submitted to GenBank as accession number HQ585874 (Supplementary Material 2 and 3). The Cd47 amplicon was ligated into the pSFFV.IRES.EGFP lentiviral vector (Fig. 1A) as described earlier (20).

Lentiviral vectors. VSV-G pseudotyped lentiviral vectors were prepared as described before (21). Briefly, lentiviral vector plasmids pSFFV.Cd47.IRES.EGFP or pSFFV.EGFP were co-transfected with plasmids psPAX2 and pMD2.G (D. Trono, Geneve, Switzerland) into HEK293T cells. Supernatants containing LV-Cd47-GFP or LV-GFP were harvested, filtered for concentration, aliquoted and stored at -80°C. Virus preparations were titrated on HepG2 cells as described earlier (22).

Contact index. 250,000 RAW264.7 macrophages were added to adherent transduced HepG2-Cd47-GFP or HepG2-GFP in a 2:1 ratio in 12 well-plates. Cells were incubated for 14 hours (37°C, 5% CO₂), RAW264.7 macrophages were then counted either as GFP⁺-cell-contacting or non-contacting. The percentage of contacting macrophages was determined for Cd47⁺ and Cd47⁻ (GFP⁺) samples. GFP-fluorescence and native light images randomly chosen across the culture areas were analyzed. For determination of the baseline, macrophages were counted from images taken 15 minutes immediately following sedimentation of the macrophages during co-culture setup (approx. 15 minutes).

Phagocytotic assay. 2.0x10⁶ TAMRA-labeled (Invitrogen) RAW264.7 macrophages were added to 5.0x10⁵ adherent HepG2-Cd47-GFP or HepG2-GFP in 2-chamber slides (Lab-Tek) together with 10 µg/ml lipopolysaccharide and kept in a FV1000 incubation system (Olympus). TAMRA and GFP fluorescence were monitored every 10 minutes for 14 hours at 31 randomly chosen locations in each the Cd47⁺ and Cd47⁻ (GFP⁺) samples. Phagocytotic events were detected as double positive (yellow) cells.

Hepatocyte lentiviral transduction and transplantation. Cryopreserved human hepatocytes (BD BioSciences) were thawed at 4°C and cell viability was measured by trypan blue exclusion. Cells were incubated with lentiviruses LV-Cd47-GFP or LV-GFP for 4 hours at 4°C at an MOI of 20. After washing with excess amounts of PBS to remove remaining free lentiviral particles, 500,000 viable hepatocytes in DMEM were transplanted into each BALB/c Alb-uPA^{tg+/+}Rag2^{-/-}IL-2Rγ^{c^{null}} mouse by intrasplenic injection, as described before (9). For each hepatocyte transduction batch, samples were kept aside in culture to determine *in vitro* initial transduction efficiencies by FACS for GFP fluorescence after 5 days. hALB levels were measured repeatedly from serum samples 4 weeks after transplantation until sacrifice according to the manufacturer's instructions (HSA Quantitation ELISA Set, Bethyl

Laboratories). All animals were maintained and handled in accordance with institutional guidelines of the Hannover Medical School and the Helmholtz Center for Infection Research, Braunschweig.

Analysis of liver sections. Successfully transplanted mice were sacrificed approximately 8 weeks after transplantation. Liver sections were fixated with fresh 4% PFA, incubated with 30% sucrose and probed with primary antibodies goat- α hALB, goat- α Cd47, rabbit- α GFP and secondary AF647-donkey- α goat and AF488-donkey- α rabbit (Supplementary Material 4) plus Hoechst-staining for cell nuclei. For microscopy, an IX81 microscope with cell^M imaging software (Olympus) and DAPI/FITC/TexasRed, CFP/YFP and Cy5 optical filtersets were used. Merged images were used for distinction of hAlb⁺GFP⁺ and hAlb⁺GFP⁻-clusters during counting.

Statistics. If not stated otherwise, data are expressed as mean $\pm$ SD of at least 3 independent experiments and the statistical significance was assessed by the Student's *t*-test. Differences were considered significant at values of $P < 0.01$.

Results

Cd47 is stably expressed in HT1080, HepG2 cell lines and primary hepatocytes after lentiviral transduction. HT1080 cells, HepG2 cells and cryopreserved human hepatocytes were transduced with the lentiviral vectors LV-Cd47-GFP and LV-GFP (control). Transcription of Cd47 and presence of hCD47 was detected in HepG2 cells and primary hepatocytes (Supplementary Fig. 2). Surface expression of Cd47 and intracellular expression of GFP was verified by FACS analysis 3 days after transduction of both HepG2 and HT1080 cells (Fig. 1B and Supplementary Fig. 5). Cd47-protein expression in transduced cryopreserved human hepatocytes and HepG2 cells was monitored by Western Blot (Fig. 1C and D).

Cd47 expression reduces recognition and phagocytosis by murine macrophages. First, we investigated the *in vitro* effects of recombinant Cd47 expressed in human hepatic cells. Murine RAW264.7 macrophages and human HepG2 cells were chosen for contact and phagocytic assays. Since *in vitro* co-culture required clean conditions without cell debris, low macrophage target cell densities and Cd47 and GFP expression at the time of experimental setup, the use of primary hepatocytes was not feasible (data not shown). The selected cell combination appeared to mimic best the *in vivo* model for transplantation of human hepatocytes. Beforehand it was important to verify that RAW264.7 macrophages expressed SIRP α and also Cd47 to ensure their derivation from a Cd47-positive mouse strain - a prerequisite for adequate Cd47-SIRP α signaling (Fig. 1E and F).

Transduced GFP-positive HepG2 cells either expressing Cd47 or not were co-cultured with RAW264.7 macrophages. Macrophages were randomly distributed amongst HepG2 cells expressing Cd47, recognized by their GFP-fluorescence, with no particular accumulation (Fig. 2A, B, E and F, and Supplementary Fig. 3, +Cd47). When incubated with HepG2 cells not

expressing Cd47, RAW264.7 showed intense clustering around the cells (Fig. 2C, D, G and H, and Supplementary Fig. 3, -Cd47). For quantification, the percentage of macrophages contacting HepG2-GFP cells with or without Cd47 expression was determined as a parameter of macrophage recognition of “non-self”. Macrophages were divided into GFP⁺-cell contacting and free macrophages. Taking into account an overall baseline of $36.5\% \pm 6.1\%$ of the contacts occurring randomly, the results demonstrated that macrophages recognize control HepG2, whereas Cd47-positive HepG2 were significantly less often in contact with macrophages (Fig. 2I).

We next analyzed if macrophage phagocytosis was also affected by Cd47 expression in HepG2 cells. RAW264.7 macrophages labeled with TAMRA were incubated with HepG2-Cd47-GFP or HepG2-GFP cells. Rare phagocytic events were monitored by immunofluorescence live imaging microscopy, being identified as double positive macrophages (Fig. 3A, B and C, and Supplementary Fig. 4). In total, approximately 6.0×10^3 macrophages co-cultured with 1.5×10^3 HepG2 were monitored in both the Cd47-GFP and the GFP groups. Thirty-five phagocytic events (*i.e.* 2.3% of HepG2) were observed in the Cd47-GFP setup and 84 (5.6% of HepG2) in the GFP control setup (Fig. 3E).

Cd47 does not enhance cell proliferation. Before conducting animal experiments, it was essential for our approach to verify that ectopic Cd47 expression does not result in a growth advantage for the Cd47⁺-subpopulation. No significant differences were observed in the proliferation rate of HepG2-Cd47-GFP and HepG2-GFP cells (Fig. 4A and B, left), thus indicating the absence of enhanced proliferation in Cd47 transgenic hepatocytes. Also, when mixing HepG2-Cd47-GFP with non-transduced HepG2, FACS analysis revealed a marginal decrease of the GFP⁺-subpopulation over a period of 14 days, whereas HepG2-GFP fractions from control mixtures remained constant, thus confirming the results from the EdU incorporation experiments (data not shown). Furthermore, proliferation assays performed with

HT1080-Cd47-GFP cells indicated no effects on proliferation due to expression of Cd47 in a fibroblast cell line (Fig. 4A and B, right).

Freshly defrosted and in vitro transduced primary human hepatocytes can be efficiently transplanted. The final step was to investigate the effect of surface expression of Cd47 on primary human hepatocytes when transplanted into BALB-ΔRAG/γ_c-uPA mice. Freshly thawed cryopreserved human hepatocytes were transduced *in vitro* with lentiviral vectors LV-Cd47-GFP, or LV-GFP as control. Following the protocol described here, preservation of cell viability was ensured and transduced cryopreserved human hepatocytes were then transplanted by intrasplenic injection (500,000 viable cells per mouse).

Samples of transduced hepatocytes of the batch used for transplantation were kept *in vitro* for 5 days (time in which the expression of transduced genes is peaking in cultured hepatocytes, data not shown). Then, GFP measurements by FACS were carried out to determine the initial transduction efficiency (see Table 1 column 6, batches A-F, and Supplementary Fig. 7).

During the lifetime of transplanted mice serum levels of secreted human serum albumin (hALB) were monitored from blood samples and only animals with hALB levels above 15 μg/ml at the day of sacrifice were regarded as successfully repopulated and included into the tissue section analyses. In total, 65% of the mice receiving Cd47-GFP transplants and 70% of GFP-only mice were included.

Mice were sacrificed 8 weeks after transplantation and tissue sections were analyzed for the presence of human cell clusters, as indicated by staining with hALB-specific antibodies. With Cd47 and Sirpα expression verified for our mouse model by RT-qPCR (Supplementary Fig. 6), liver sections were stained for endogenous and transduced Cd47 (Fig. 5A-E).

Analysis of chimeric liver sections reveals a strong protective effect of Cd47 expression. To evaluate whether engraftment of the subpopulation of cells expressing Cd47-GFP within a

sample of transplanted cryopreserved human hepatocytes is favored, we performed immunofluorescence co-staining for hALB and GFP of liver sections from sacrificed mice, analyzing and counting individual GFP-overlays of hALB⁺-clusters (Fig. 6C-J, and Supplementary Fig. 8). Liver sections from transplantations of hepatocyte samples containing GFP-only subpopulations served as a control. All liver lobes from a sacrificed mouse were examined to obtain a representative picture on the global distribution of human clusters expressing hAlb/GFP or hAlb only. Great care was also taken to ensure that individual sections were at a distance from each other to exclude the possibility of double-counting clusters. The number of GFP expressing clusters was divided by the total number of hALB-positive clusters in the sections analyzed, and expressed as percentages (Fig. 6A, and Table 1 column 5). The use of the total number of hALB⁺-clusters as a crucial internal baseline value facilitated independency from individual repopulation rates - a necessity given the commonly experienced substantial variation in engraftment levels between individual mice. The percentage of lentivirally transduced - thus expressing Cd47-GFP or GFP only - clusters within the total population of hALB⁺-clusters (Table 1 column 5) was finally divided by the corresponding initial transduction percentages, as defined by FACS analysis of the *in vitro* cultured samples that were preserved after transduction of the hepatocytes (Fig. 6B, Supplementary Fig. 7, and Table 1 column 6), thereby resulting in a selection ratio calculated for each mouse (Table 1 column 7). Selection ratios above 1.0 indicate an *in vivo* enrichment of transduced hepatocytes within the total population of transplanted cells, whereas ratios below 1.0 indicate a selective *in vivo* loss of transduced cells.

Fig. 6K summarizes data for mice successfully transplanted with LV-Cd47-GFP- and LV-GFP-transduced hepatocytes, respectively. The comparison of selection ratios reveals that the proportion of Cd47-expressing hepatocytes (BD-Cd47-GFP) has doubled *in vivo* (see also Table 1 column 7: average selection ratio 2.0), even more when compared to the proportion of hepatocytes not expressing Cd47 (BD-GFP, average selection ratio 0.4). Overall, the findings

strongly indicate a selective advantage for human primary hepatocytes ectopically expressing Cd47.

Thus, our data demonstrate that ectopically expressed Cd47 not only marks human liver cells for murine macrophages as "self" *in vitro*, but also *in vivo* leads to durable engraftment and strong enrichment of Cd47-positive hepatocytes.

Discussion

Most, if not all, of the immunodeficient mouse strains used for the generation of human-mouse chimeric livers still develop macrophages as the last remaining major population of the immune system. These macrophages reject human xenografts to varying extents, depending on the SIRP α -allele present in the particular mouse strain (23). With the aim to facilitate the generation of chimeric livers, we investigated whether the species-specificity of the CD47-SIRP α system for the recognition of "self" can be used to down-modulate murine macrophage activity against human Cd47-expressing primary hepatocytes.

In a series of *in vitro* experiments using a human hepatic cell line lentivirally transduced for the expression of Cd47, we could show a significant decrease of counted contacts with murine macrophages in Cd47-expressing liver cells as compared to Cd47-negative controls. This indicates macrophage recognition of "self" and consequently shorter periods of cell-cell contacts. Furthermore, the number of phagocytic events was reduced by more than 50% when human cells were protected by Cd47.

For *in vivo* hepatocyte transplantations into BALB- Δ RAG/ γ_c -uPA mice, freshly thawed cryopreserved human primary hepatocytes were used. Since primary hepatocytes are only efficiently transplantable directly without *in vitro* culture (9), we developed a protocol to lentivirally transduce thawed cells when still cold and immediately before transplantation. For transduction with the lentiviral vector, a MOI was chosen that statistically ensured the presence of only one vector copy per cell to avoid negative effects of multiple integration events which would result in excessive Cd47 and/or GFP expression in transduced cells. Therefore, transduction efficiencies were adjusted to a range between 4-25% of the cell population. In addition, this range provided sufficient resolution for accurate detection of the hypothesized increases in GFP⁺-cluster numbers.

In this approach, freshly transduced and immediately transplanted primary hepatocytes were not expressing significant amounts of Cd47 in the following 2-3 days until protein expression from the integrated lentiviral vector set in. Thus, there was no potential protection against macrophages in the early stages of the transplantation experiments. However, to the advantage for our proof-of-principle experiments, the delayed protein expression excluded the possibility that Cd47 could interfere with other processes (like hepatocyte migration or passage of the liver sinusoidal endothelium, etc.) preceding engraftment in the liver parenchyma. In future cell xenotransplantation experiments - then heading for maximal repopulation - this initial vulnerability to macrophage action may be overcome by a single dose - and therefore minimised adverse effects (24) - of clodronate prior to transplantation (7). Two months after cell transplantation, livers from mice were harvested. Liver sections were examined for GFP-positive cell clusters amongst the whole hALB-positive cluster population. Percentages of GFP-positive clusters were compared to initial percentages in the not transplanted, transduced samples that had been kept in culture for FACS analysis.

Surprisingly, GFP-positive clusters in the GFP-only transplantation group appear underrepresented when compared to the initial *in vitro* transduction efficiencies of the respective cell batch, resulting in selection ratios lower than 1. Since cytotoxic effects of GFP-expression in hepatocytes can almost certainly be excluded given common experiences with GFP, we can only speculate about the reasons for this seemingly *in vivo* loss of transduced cells. Most likely, the transducing lentiviruses exhibit a slight preference for hepatocyte subpopulations in the preparations that survive five days in tissue culture but are not the most suitable subpopulations for efficient repopulation of liver tissue. However, this effect should also be valid for the Cd47-GFP-transduction group and thus it seems reasonable to assume that the real advantage of ectopic Cd47 expression in our system is underestimated by our data. Furthermore, higher initial transduction efficiencies - providing the denominator in the fraction calculating the ratio - result in decreased selection ratios and vice versa solely

for mathematical reasons. With higher transduction efficiencies found in the Cd47-GFP group, the real Cd47-effect again tends to be underestimated. Even without taking these effects into account, we see a double number of engrafted human cell clusters in the murine liver when cells are expressing Cd47 (selection ratio 2.0).

Since the macrophages identified in tissue sections were consistently too low in numbers for quantitative analyses (data not shown), we could not directly demonstrate macrophage-hepatocyte interactions *in situ*. Therefore, we are also not able to conclude whether the effect seen is caused by liver resident (Kupffer cells) or systemically circulating macrophages. Our transplantation experiments do not allow us to exclude also the possibility that other functions of the Cd47 protein might confer the selective advantage observed. None of the known functions of CD47 (25-29) appears to be a reasonable alternative to down-regulation of macrophage activity through SIRP α in our mouse model deficient of B- and T-cells.

Murine macrophage rejection of human xenografts is not absolute and of variable severity dependent on the strain background of the immunodeficient mouse model used for xenotransplantation. SIRP α polymorphism is one of the factors responsible for the variable degrees of rejection observed between mouse strains (23). The simple, ectopic and dominant expression of murine Cd47 in human cell transplants will most likely also reduce the need for laborious and time-consuming back-crossings of mouse model properties into strain backgrounds with more tolerogenic murine SIRP α alleles and thus down-regulated macrophage activity. Alternatively, animals transgenic for human SIRP α can be used as recipients, rendering ectopic expression of Cd47 dispensable (30).

Taken together we are able to demonstrate the capability of ectopically expressed murine Cd47 to improve humanization of the liver by at least 100%. Our results qualitatively confirm in a solid organ the findings of Legrand *et al.*, who used a similar approach to facilitate engraftment of human hematopoietic cells in mice (19). Since achieving optimal humanization levels in a small animal model is critical for many applications in basic and

preclinical medical research, our straightforward approach of protecting transplanted xenogeneic cells through lentivirally introduced Cd47 might turn out to be a helpful breakthrough towards the goal of generating robust humanized animal models.

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Figure Legends

Fig. 1. Expression of Cd47 and SIRP α . (A) Vector map of pSFFV.Cd47.IRES.EGFP. An internal ribosome entry site (IRES) ensures co-expression of GFP and Cd47. (B) FACS analysis of Cd47 and GFP expression. Cd47 and GFP show clearly detectable peaks in HepG2-Cd47-GFP. HepG2-GFP cells do not stain for Cd47 but express GFP. (C) Western blot of primary human hepatocytes transduced with LV-Cd47-GFP (+Cd47) or LV-GFP (-Cd47) as control. Cd47 is expressed as a ~50 kDa band and is heavily glycosylated in primary hepatocytes. (D) Western Blot of HepG2 cells transduced with LV-Cd47-GFP (+ Cd47) or LV-GFP (- Cd47) as control. (E) FACS analysis of RAW264.7 macrophages for SIRP α and (F) endogenous Cd47 expression. RAW264.7 macrophages express both SIRP α and Cd47.

Fig. 2. Contact assay. RAW264.7 murine macrophages were added to adherent GFP-positive HepG2 cells expressing Cd47 (A, B, E and F) or not (C, D, G and H). Macrophage aggregation was monitored by light (B, D, F and H) and matching fluorescence (A, C, E and G) microscopy. RAW264.7 macrophages incubated with GFP-positive HepG2 cells expressing Cd47 (A and E) show random scattering with no or minimal accumulation around HepG2 cells (B and F). In contrast, RAW264.7 macrophages show intense accumulation (D and H) around GFP-positive HepG2 cells not expressing Cd47 (C and G). Scale bars: (A-D), 200 μ m; (E-H), 100 μ m. Results from counting a total of 4135 macrophages (+Cd47: 2033 macrophages, -Cd47: 2102) (I): The percentage of RAW264.7 cells in contact with GFP⁺-HepG2 cells expressing Cd47 or not was calculated and served as contact index. The baseline of 36.5% $\pm$ 6.1 (horizontal lines) of contacts occurring solely by chance was calculated from contact-counting of 1446 macrophages immediately after co-culture setup. HepG2-Cd47-GFP cells are contacted significantly less often than control HepG2-GFP cells. n = 20, Error bars: SD.

* indicates cluster of RAW264.7 macrophages on HepG2 cells not expressing Cd47

Fig. 3. Phagocytosis of HepG2 cells. Time-lapse microscopy of GFP-positive HepG2 cells expressing Cd47 (D, + Cd47) or not (A, B and C, - Cd47), incubated with TAMRA-labeled RAW264.7 macrophages (red). RAW264.7 phagocytosis was more often observed with control HepG2 cells (A, B and C) (red arrows) than with Cd47⁺-HepG2 cells (D). (B and C) show magnified areas with examples of phagocytic events. The total numbers of detected phagocytoses are displayed in (E). Scale bars: 100 μ m.

Fig. 4. Proliferation of Cd47 expressing cells. (A) EdU incorporation, indicative of cell proliferation in transduced HepG2 and HT1080 cells expressing Cd47-GFP or GFP-only as control. HT1080 cell proliferation was not affected by overexpression of Cd47, whereas HepG2 cells showed marginally decreased proliferation rates when expressing Cd47. Error bars: SD. (B) FACS data from EdU proliferation assay of HepG2 and HT1080 cells expressing Cd47-GFP or GFP.

Fig. 5. Staining of liver sections from transplanted BALB- Δ RAG/ γ_c -uPA mice. Liver sections from mice 37501 (A-C) and 37505 (D-F) were stained for GFP, cell nuclei (Hoechst) and either Cd47 (A-E) or hALB (F). In Cd47-GFP transduced human hepatocytes, transmembrane location of Cd47 is seen (B, red arrows). Endogenous murine Cd47 (C-E, areas m) as well as transduced Cd47 are detectable, generating a sharp border (C, red arrows). (F) Two types of human hepatocytes, non-transduced hALB⁺ GFP⁻ and transduced hALB⁺ GFP⁺ are detectable: Staining for hALB in a neighbouring section (10 μ m apart from D and E) identifies both transduced (+ and GFP-positive) and non-transduced transplant types (-). Scale bars: A, D, F 250 μ m; B, C 50 μ m; E 113 μ m.

Fig. 6. Transplantation of human hepatocytes into BALB- Δ RAG/ γ_c -uPA mice. uPA mice were transplanted with Cd47-GFP or GFP-only transduced hepatocytes. After 8 weeks liver sections were stained for human albumin (hALB) and GFP. (A) Overlay of one exemplary complete liver lobe section from the mouse 37505 transplanted with Cd47-GFP hepatocytes. The image was assembled from a total of 110 individual images (see F and J) of one whole section for demonstration purposes. Arrows indicate clusters identified (yellow: hAlb-positive, green: hAlb/GFP-positive). The percentage of GFP⁺-clusters within all hALB⁺-clusters from all sections of mouse 37505 is 31.6%. (B) *In vitro* FACS analysis of hepatocytes transduced with LV-Cd47-GFP 5 days after transduction, yielding 21.2% GFP-positive cells in transduction batch C (see Table 1). Cell aliquots for FACS were kept in culture in parallel to aliquots used for immediate transplantation after transduction. (C-J) Individual cluster analyses: cell clusters were analyzed by staining for hALB (C and G), GFP (D and H), cell nuclei (E and I) and overlaid (F and J). (K) Overall summary (see Table 1 column 7), with selection ratios below 1.0 indicating selective loss and above 1.0 selective enrichment of transduced hepatocytes *in vivo*. A red arrow highlights the result from the calculations for exemplary mouse 37505. Horizontal lines indicate mean values (Table 1, average Cd47-GFP⁺: 2.0 and GFP⁺: 0.4). Scale bars: 250 μ m.

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6