Anti-inflammatory liposomes have no impact on liver regeneration in rats

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HIGHLIGHTS

- Use of anti-CD163-dexamethasone is an attractive strategy for anti-inflammatory treatment.
- In the present study the impact of anti-CD163 dexamethasone on liver regeneration in rats was studied.
- We show that low dose anti-CD163 dexamethasone has no negative effect on liver regeneration after 70% hepatectomy in rats. Characters should then be down to 122.

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ABSTRACT

Introduction: Surgical resection is the gold standard in treatment of hepatic malignancies, giving the patient the best chance to be cured. The liver has a unique capacity to regenerate. However, an inflammatory response occurs during resection, in part mediated by Kupffer cells, that influences the speed of regeneration. The aim of this study was to investigate the effect of a Kupffer cell targeted anti-inflammatory treatment on liver regeneration in rats.

Methods: Two sets of animals, each including four groups of eight rats, were included. Paired groups from each set received treatment with placebo, low dose dexamethasone, high dose dexamethasone or low dose anti-CD163 dexamethasone. Subsequently, the rats underwent 70% partial hepatectomy. The two sets were evaluated on postoperative day 2 or 5, respectively. Blood was drawn for circulating markers of inflammation and liver cell damage; liver tissue was sampled for analysis of regeneration rate and proliferation index.

Results: The high dose dexamethasone group had significantly lower body and liver weight than the placebo and anti-CD163-dex groups. There were no differences in liver regeneration rates between groups. Hepatocyte proliferation was completed faster in the placebo group, although this was not significant. The anti-CD163-dex group showed increased blood levels of albumin and alanine amino-transferase and a diminished inflammatory response in terms of significantly reduced haptoglobin, α2-macroglobulin and Interleukine-6.

Conclusion: Low dose dexamethasone targeted to Kupffer cells does not affect histological liver cell regeneration after 70% hepatectomy in rats, but reduces the inflammatory response judged by circulating markers of inflammation.

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1. Introduction

The liver has a striking regenerative capacity after hepatic resection. Postoperative regeneration is mediated through an orchestrated proliferation of hepatocytes (not involving stem cells), resulting in enlargement of the residual liver lobes with restoration of liver mass [1]. This process is carried out at the same time as the liver maintains support for body homeostasis [1].

Surgical resection or transplantation, are the only potentially curative treatment options for most cases of hepatic malignancy [2]. Thus, the possible demand for therapeutic hepatectomy is enormous. For example, colorectal cancer is the third most prevalent cancer in the world [2]. Almost half of these patients will develop colorectal liver metastases during the course of their disease [3–5]. Untreated, their median survival is only 10 months [4], whilst 5-year survival is very rare [3,6]. Similarly, primary liver cancer (in most cases hepatocellular carcinoma) is globally the second most frequent cause of cancer death in men [7]. Again, surgery remains the only curative treatment for most cases.

Perioperative blood loss and transfusion requirements are factors known to be associated with the degree of morbidity and mortality following hepatic resection [8,9]. Vascular occlusion techniques such as Pringle's maneuver may be used in an attempt to limit hemorrhage [9]. However, the ischemic effect of this type of maneuver may induce a harmful inflammatory response known as ischemia-reperfusion (IR) injury [9,10].

From previous studies, Kupffer cells are known to be involved in the activation of cell division during the process of liver regeneration by the production of tumor necrosis factor (TNF)-α and interleukin (IL)-6 [1,11,12]. However, Kupffer cells are also known to be mediators of IR injuries [13–15].

Considerable research has been conducted on different approaches to enhance liver survival and viability after resection [9,12]. Focus has mainly been on surgical procedures such as ischemic conditioning [16] and intermittent clamping [17]. However more recently, attention has been directed at pharmacological strategies and positive results have been reported suggesting a protective effect of treatment with sevoflurane [18] and with prednisolone [19,20]. Against this backing, a possible new strategy could be anti-inflammatory therapy with the novel reagent anti-CD163-mAb conjugated PEGylated liposomes [21] that can be loaded with dexamethasone to form anti-CD163-dex-lipo. Anti-CD163-dex-lipo is directed against the CD163 receptor. CD163 is a hemoglobin scavenger receptor, highly expressed on macrophages in the liver, spleen and bone marrow, and at sites of inflammation [22].

Previously, it has been shown that anti-CD163-dex constructed as an antibody-drug conjugate (ADC) inhibits the inflammatory response of rat macrophages after injection with lipopolysaccharides (LPS) [22]. In addition, we showed that anti-CD163-dex ADC protects against IR injury after liver ischemia by inhibiting apoptosis [23]. The aim of the present study was to investigate the possible effect of Kupffer cell targeted anti-inflammatory treatment with anti-CD163-dex liposomes on liver regeneration in rats after partial hepatectomy (PHx).

2. Material and methods

2.1. Animals and ethics

All animal experiments were performed under the approval of Danish Animal Experiment Inspectorate, Copenhagen, Denmark (license number 2012-15-2934-00591 expansion), and in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institute of Health, USA [24]. Male Wistar rats of 200 g, corresponding to an age of app 50 days, were obtained from Taconic Biosciences (Borup, Denmark) and were acclimatized for one week prior to operation. The animals were housed in standard animal laboratories with the temperature maintained at 23 °C, an artificial 12 h light-dark cycle, and free access to food (Altromin) and water. The rats were daily monitored with regard to weight, behavior and physical appearance by veterinary nurses and the first author. All animals were daily scored by humane endpoint with 'General Distress Score' as described by Lloyd and Wolfensohn [25]. Briefly the rats were evaluated on the following endpoint: Appearance; Food and water intake; Clinical signs; Natural behavior and Provoked behavior. If a score of 2 was observed in any parameter the animals were even closer observed and attended to at minimum 8th hour intervals. If the condition was not remedied during 24 h the rat was euthanized by cervical dislocation. If a score of 3 was observed in any parameter the animals were euthanized immediately. Dead rats were autopsied to establish the cause of death. The species, sex and size/age, was chosen based on previous experience in the research group regarding studies in liver regeneration in rats [26,27].

2.2. Experimental design

2.2.1. Design

Sixty-four rats were given treatment, underwent 70% PHx, and were evaluated on postoperative day (POD) 2 or 5 (Fig. 1). The choice of evaluation days was based on a previously conducted study, which showed day 2 and 5 to be key points in rat liver regeneration [26].

2.2.2. Treatment

With the operator blinded to treatment, the animals were block randomized into four groups. They received either 1) placebo (phosphate buffered saline), 2) low dose dexamethasone-phosphate (LDD; 0.2 mg/kg), 3) high dose dexamethasone phosphate (HDD; 1.0 mg/kg), or 4) Anti-CD163-mAb conjugated liposome-encapsulated low dose dexamethasone-21-hemisuccinate (anti-CD163-dex; 0.2 mg/kg). The synthesis of anti-CD163-liposomes has been described previously [21] and remote loading with dexamethasone-21-hemisuccinate was performed as described in Ref. [28]. The conjugate was administrated by intravenous injection (2 ml/kg) to the tail vein 18 h before liver resection. For animals evaluated on POD 5, an additional dose of treatment was given at POD 2. The doses used in the present study, were based on experience from earlier studies conducted on dexamethasone and anti-CD163-dexamethasone [22,29].

2.2.3. Anesthetics and analgesia

General anesthesia with sevoflurane was used during administration of treatment, surgical procedures and at euthanisation. The animals were anesthetized in an induction chamber with a mixture of oxygen (2.0 L/min), N_2O (0.5 L/min) and 4% sevoflurane (Forene; Abbott Laboratories, Maidenhead, UK). During procedures, anesthesia was maintained with 3% sevoflurane in oxygen and N_2O as described above, which was administered through a mask covering the face of the rat. Before surgery, the animals were given a subcutaneous injection of a long-lasting non-steroid anti-inflammatory drug, 5 mg/kg Carprofen (RimadylVet; Pfizer Animal Health, Exton, USA) and 2.5 ml of isotonic saline. Injection of analgesics was repeated on POD 1, 2, and 3.

2.2.4. Surgical procedure

The animal was placed in a supine position on a temperature controlled heating pad, a midline abdominal incision was made and the liver was mobilized. PHx was performed as first described by *Higgins and Anderson* [30]. In brief, the median and left lateral lobes
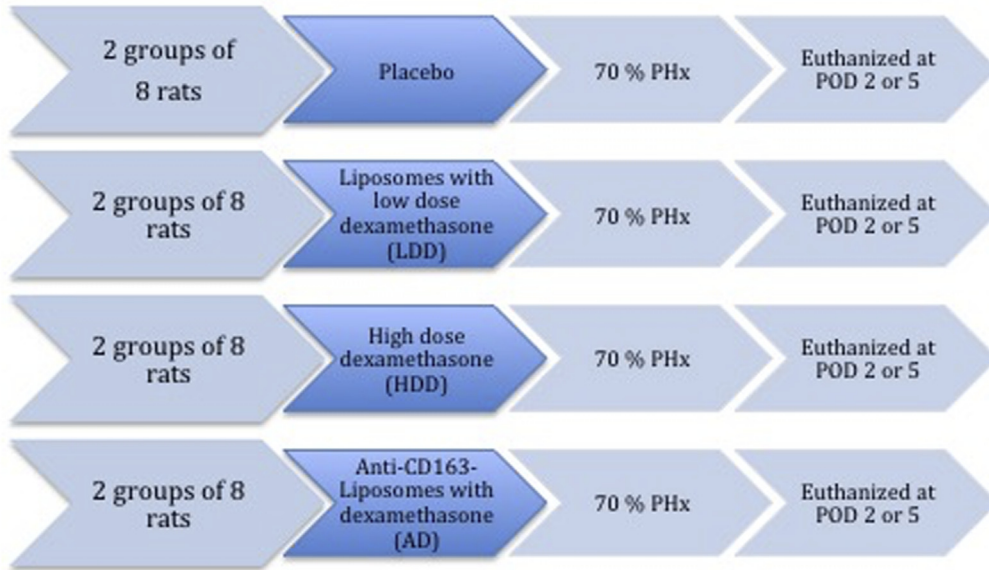
Fig. 1. Flowchart. The flowchart of the experiment.

of the liver were ligated and the lobes were resected, resulting in 70% liver resection. The abdominal wall was then closed with a 4−0 absorbent suture and the skin closed with agraffes.

2.2.5. Evaluation

After PHx, the rats were again block randomized, this time into two sets of four treatment groups for evaluation on either POD 2 or 5. On the given POD, the rats were anaesthetized, blood samples were collected from the heart by cannulation and they were then euthanized by cervical dislocation. The regenerated liver was removed, weighed and the caudate liver lobe used for histological assessment.

2.3. Biochemical analysis

Blood was sampled from the heart at euthanisation, processed and stored at −80 °C until analysis. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, albumin and haptoglobin levels were measured using Cobas 6000 (Roche Diagnostics, Hvidovre, Denmark). Serum levels of IL-6 and TNF-α were determined using commercial enzyme-linked immunosorbent assays (ELISAs; Life Technology, Naerum, Denmark). Rat acute phase protein alpha-2-macroglobulin (α2M) was detected using a quantitative ELISA-based test kit (Consultants Laboratory, Portland, USA). All assays were performed as specified by the manufacturers' instructions.

2.4. Liver weight and regeneration rate

The change in liver weight was evaluated as the hepatic regeneration rate (RR). RR was defined as: liver weight per 100 g of body weight at euthanisation divided by preoperative calculated liver weight per 100 g of body weight multiplied by 100

$$RR = \frac{\left(\frac{LWm}{100gBW}\right)sac}{\left(\frac{LWc}{100gBW}\right)pre} \cdot 100$$

LWm is the measured liver weight at euthanasia and LWc is the preoperative calculated liver weight. The preoperatively estimated total liver weight was calculated from the resected liver weight. After removing 70% liver tissue the LWc was estimated as 100%: $LW_C = $ (Weight of 70% rec/70) x 100.

2.5. Histological evaluation

2.5.1. Tissue preparation

For histological evaluation the caudate liver lobe was fixed in phosphate-buffered 4% formaldehyde, cut into 2 mm parallel slabs using a tissue slicer, and embedded in paraffin. A 30-μm section was cut from each of the paraffin-embedded blocks for immunohisto-chemical staining.

2.5.2. Immunohistochemistry

Hepatocellular proliferation was estimated using immunohisto-chemical staining for the Ki-67 antigen. Ki-67 is expressed during all active phases of the cell cycle (G1, S, G2 and M phases), but not in resting (G0) cells [31]. Immunohistochemical staining of the 30-μm paraffin sections was performed on an automatic stainer using a standard (in-house) protocol, modified for use in thick sections, as described previously [26]. In brief, following epitope retrieval the sections were stained with monoclonal mouse anti-rat Ki-67 spe-cific antibody (Dako, Glostrup, Denmark), bound antibody was detected and positive signals visualized by using the EnVision+ horseradish peroxidase labeled anti-mouse detection system (Dako), and the sections were counterstained with hematoxylin.

2.5.3. Stereological quantitation

Stereological methods were applied to quantify the number of proliferating hepatocytes in the immunohistochemically stained sections [32]. The liver sections were prepared according to ste-reological principles using systematic uniform random sampling (SURS) [33,34]. All sections were analyzed using the Olympus BH-50 microscope with a 60x oil objective lens. The microscope was modified for stereology with a motorized stage and a digital camera connected to a PC with newCAST 3.6.5.0 software (Visiopharm, Hørsholm, Denmark). This gave a magnification of 3014x. The same investigator analyzed all sections and was blinded to the treatment group.

The optical fractionator method [35,36] was used to assess the total number of Ki-67-positive cells in each rat liver. Approximately 0.5% of the section area was analyzed for Ki-67-positive cells and the thickness of the sampled section was measured every time a positive cell was seen in the frame. The number of Ki-67-positive cells N was calculated using the following equation:

$$N = \frac{1}{SSF} \cdot \frac{1}{ASF} \cdot \frac{1}{HSF} \cdot \sum Q^-$$

SSF is the Section Sampling Fraction $= t/T$, t is the height of the sampled section cut on a calibrated microtome (30 μm), and T is the average thickness of the embedded slabs following shrinkage. The thickness of the slabs was 2 mm before embedding. Several of these were exhaustively cut in thin histological slices to measure slab thickness after shrinkage. Seven tissue landmarks for these slabs were followed through the whole series of sections and the average slab thickness after shrinkage was calculated:

$$T = (1510 + 1570 + 1585 + 2005 + 2080 + 1885$$
$$+ 1850) \, \mu m/7$$
$$= 1784 \, \mu m$$

ASF is the Area Sampling Fraction $= \frac{a(frame)}{dx \cdot dy}$, a(frame) is the area of the 2D unbiased counting frame, 7500 μm^2, and dx and dy are the stepping distance in the x- and y-direction equal to 1225 μm, respectively.

HSF is the Height Sampling Fraction $= \frac{h}{t(Q^-)}$, where h is the disector height (15 μm) and t(Q$^-$) is the number(Q$^-$) weighted mean section thickness (=15/28.32).

Q-is the number of positive cells counted. The counting rules used are described in Fig. 2.

2.6. Statistical analysis

All statistical analyses were performed in SPSS 20 for Windows (SPSS Inc., Chicago, Illinois, USA). Data were presented as mean (±standard error (SE)) and p-values < 0.05 considered significant.
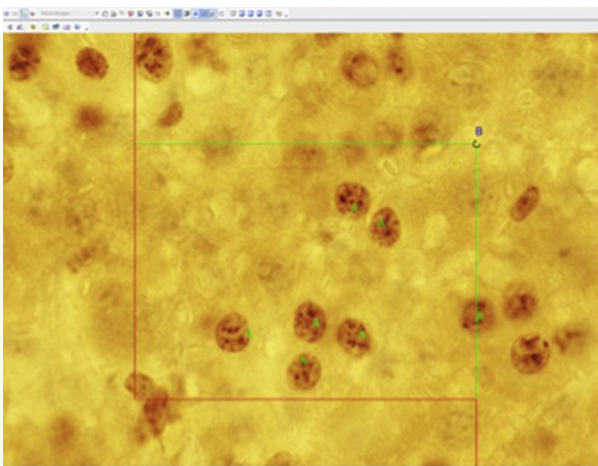
Fig. 2. Immunohistochemical staining of liver section for Ki-67 proliferation antigen, including counting frame. The counting frame (100 × 75 μm) consists of inclusion lines (green lines seen to the right and in the upper part of the frame) and exclusion lines (red lines to the left and in the lower part of the 2D frame, together with lines at the top and bottom of the disector height). Positively stained hepatocytes were counted if their nuclei were in focus inside the counting frame or if they touched an inclusion line, and did not touch the exclusion lines. Seven Ki-67 positive hepatocytes (marked with A's) can be seen in this frame.

To determine if there were any significant differences between the groups, a one-way ANOVA test was performed when applicable. One-way ANOVA was considered applicable if data met the assumptions of no outliers, were normally distributed and had homogeneity of variances. Nonparametric data were tested by Kruskal−Wallis test followed by Mann−Whitney U test.

3. Results

3.1. Mortality

Ten animals died before evaluation; three from the placebo group (two from infection and one from an unidentifiable cause), two in the LDD group (from infection), four in the HDD group (from infection), and one in anti-CD163-dex group (as a consequence of wound rupture resulting in intestinal perforation).

3.2. Body weight

The animals had a mean body weight of 202 g (191−217 g) before medication and surgery. In the HDD group, there was a steady decline in body weight throughout the experiment, this being significantly lower at all time points compared with the placebo (before res p < 0,0005; POD2 p = 0.008; POD5 p = 0.001) and to the anti-CD163-dex (before res p < 0.002; POD2 p = 0.003; POD5 p = 0.004) groups (Fig. 3a). On POD 2, the anti-CD163-dex group was significantly heavier than the LDD group (p = 0.012) (Fig. 3a). On POD 5, the body weight was also significantly lower in the HDD group compared with the LDD group (Fig. 3a).

3.3. Liver weight

The average removed liver weight was 6.26 g (5.26−7.30 g) (mean of all groups on resection time, 18 h after treatment). The mean liver weight of the HDD group was significantly lower than the placebo group on POD 5 (p = 0.002) (Fig. 3b).

3.4. Liver regeneration rate

On POD 2, there was a trend towards a lower regeneration rate in the HDD group compared with the placebo group. However this difference was not significant (p = 0.062). Otherwise, regeneration rates were comparable between the groups (Fig. 4).

3.5. Hepatocyte proliferation

There were no significant differences in the hepatocyte proliferation between the groups on POD 2 (Fig. 5). On POD 5, the proliferation was significantly lower in the placebo group compared with the anti-CD163-dex (p = 0.015) and LDD (p = 0.015) groups (Fig. 5).

3.6. Biochemistry

On POD 2, ALP was significantly lower in the HDD group compared with the placebo group (p = 0.042) (Table 1). On POD 5, ALT was significantly higher in the anti-CD163-dex group compared with placebo (p = 0.038) and albumin was significantly higher in the anti-CD163-dex group compared with the HDD group (p = 0.02) (Table 1). On POD 5, haptoglobin (p = 0.02), α2M (p = 0.038) and IL-6 (p = 0.038) were significantly lower in the anti-CD163-dex group compared with the HDD group (Table 1). In addition, α2M was significantly lower in the anti-CD163-dex group compared with the placebo group (p = 0.028) on POD5 (Table 1).
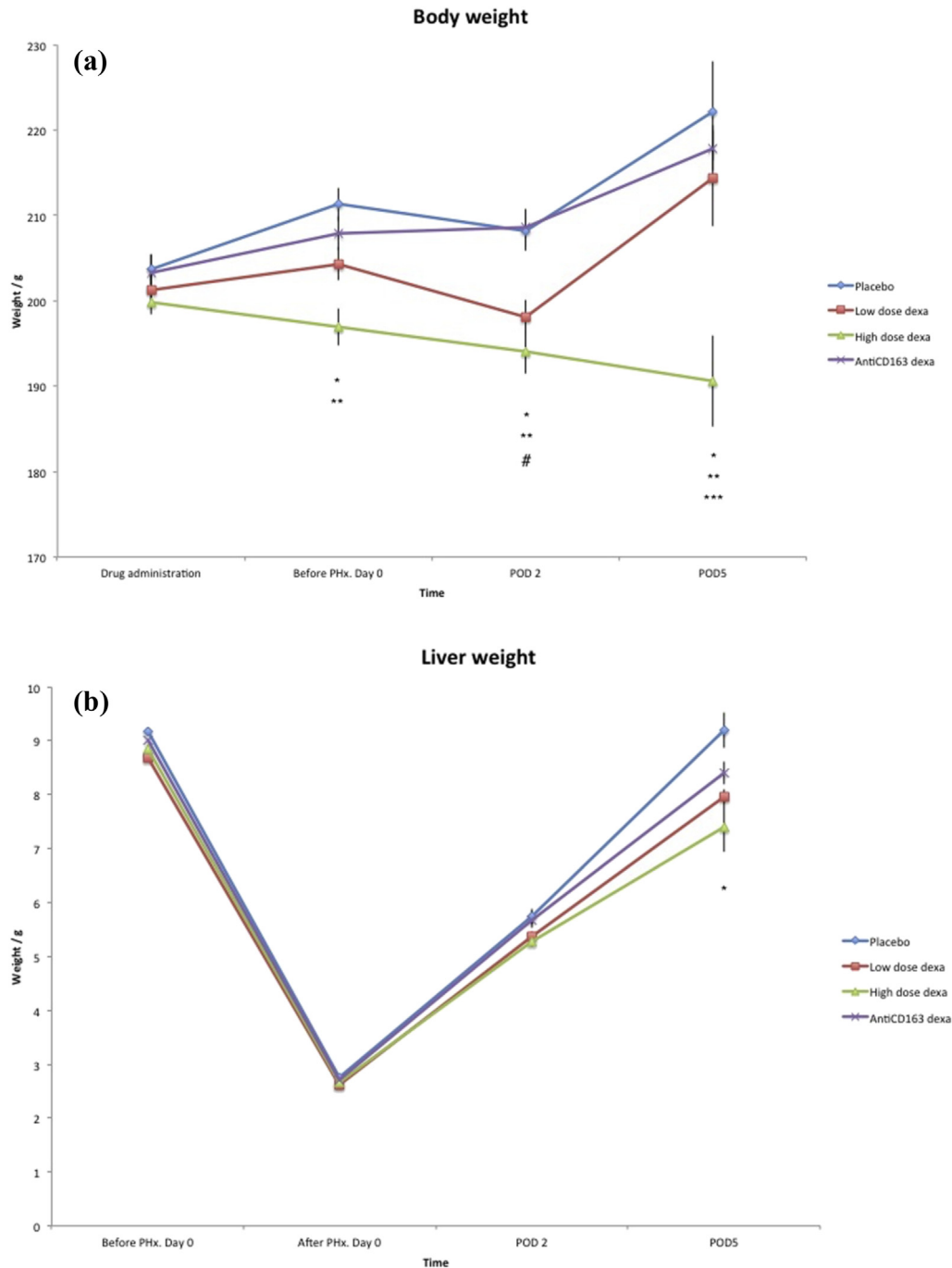
Fig. 3. **a**. **Body weight**. The dynamics in body weight. Mean (±SE). Significant differences have been indicated with symbols. Before resection day 0: HDD vs. placebo (*p < 0,0005) and HDD vs. anti-CD163-dex (**p = 0.002). At POD 2: HDD vs. placebo (*p = 0.008); HDD vs. anti-CD163-dex (**p = 0.003) and anti-CD163-dex vs LDD (#p = 0.036). At POD 5: HDD vs. placebo (*p = 0.001), HDD vs. anti-CD163-dex (**p = 0.004) and HDD vs. LDD (***p = 0.012). **b**. **Liver weight**. The dynamics in liver weight. Mean (±SE). Significant differences have been indicated with symbols. POD 5: HDD vs. placebo (*p = 0.002).

4. Discussion

We investigated the possible effect of a Kupffer cell targeted anti-inflammatory treatment on liver regeneration in rats. Our study found that treatment with the low dose antibody conjugate anti-CD163-dex before PHx had no apparent effect on histological post-resection liver regeneration, compared with controls treated with other regimens or placebo. These results require further testing in future studies. However, they suggest that anti-CD163-dex treatment may be applied without undue concern that the

antibody conjugate might be associated with undesirable anti-proliferative effects on the liver after resection.

Kupffer cells are believed to be the principal source of cytokine release after PHx. Cytokines are key mediators that may initiate, modulate, or suppress liver regeneration after hepatectomy [13—15,37]. However, there is also strong evidence that they play a central role in triggering the undesirable inflammatory reactions involved in IR injury. Use of anti-CD163-dex is an attractive strategy for anti-inflammatory treatment, as it may allow the specific de-livery of a localized anti-inflammatory effect targeted through the
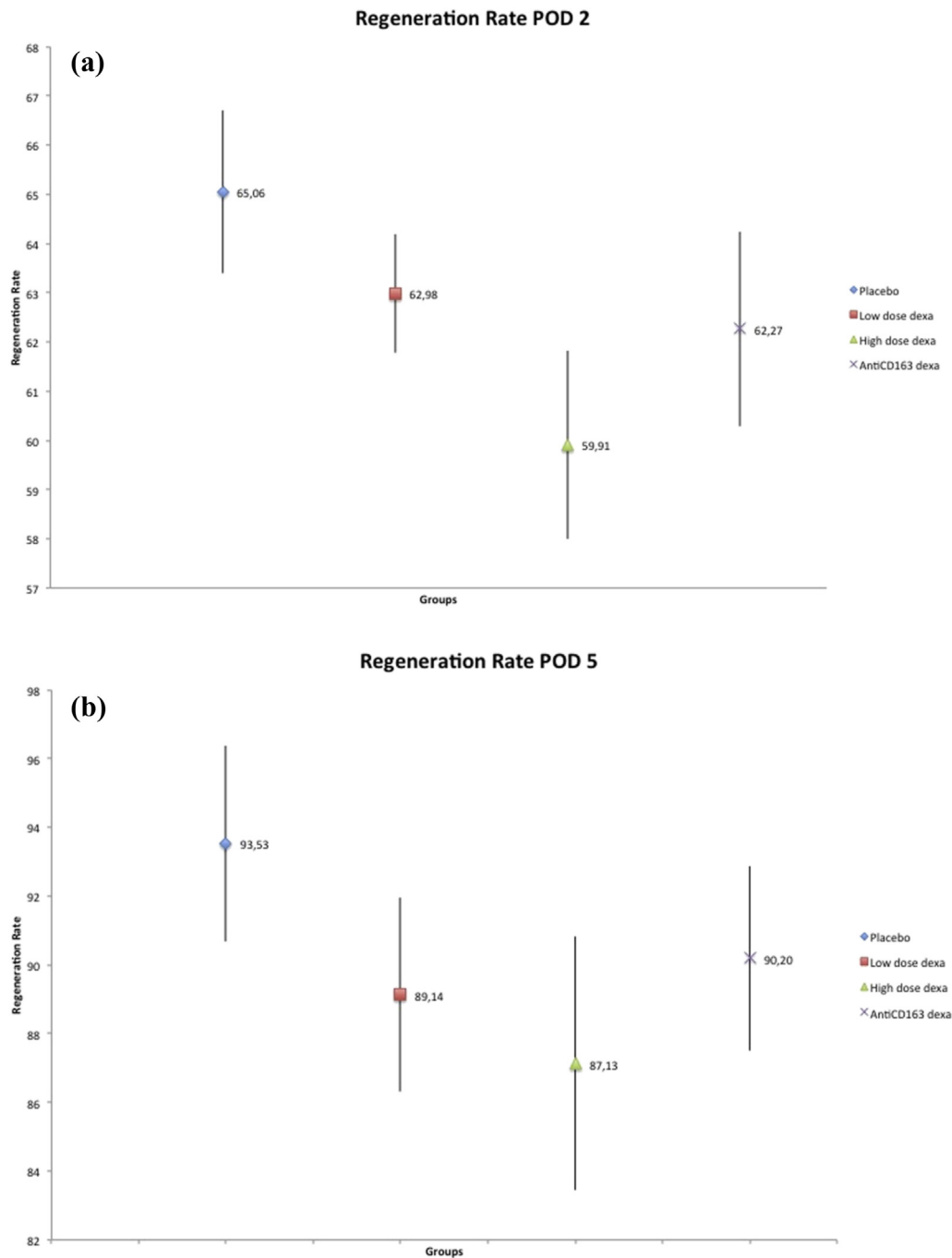
Fig. 4. (**a**+**b**). **Regeneration Rate.** The regeneration rate on POD 2 and POD 5. Mean ($\pm$SE).

macrophage-specific CD163 receptor, whilst potentially avoiding the often, serious systemic effects of high-dose glucocorticoids.

We found no differences in either liver weight or regeneration rates comparing rats treated with placebo or with anti-CD163-dex, on either POD 2 or POD 5. In a previous study, Meijer et al. used experimental administration of clodronate-loaded liposomes (CI$_2$MDP) to deplete cytokines through the complete physical elimination of Kupffer cells [37]. This led to inhibition of liver regeneration, as judged by regeneration rate. Moreover, Meijer et al. showed that CI$_2$MDP treatment inhibited hepatocyte DNA synthesis in the regenerating liver, as determined by the hepatocyte bromodeoxyuridine (BrdU) labeling index. Given the evidence that Kupffer cell activation is important in post-resection hepatocyte

proliferation the present and the study by Meijer et al. are in agreement as anti-CD163-dex did not totally abolish the cytokine response as did CI$_2$MDP treatment [37]. We found a significant higher hepatocyte proliferation in the LDD group compared to placebo, but no significant difference between the placebo and the HDD group. The latter was probably due to a higher variance in the HDD group than the LDD group regarding this parameter.

Tsutsumi et al. studied the effect of selective inhibition of TNF-α and IL-6 on liver regeneration in rats receiving a 90% hepatectomy. In contrast to Meijer et al. [37], Tsutsumi et al. found that suppression of cytokines (TNF-α and IL-6) stimulated liver regeneration as judged by proliferating cell nuclear antigen (PCNA) and by remnant liver weight [38]. They also demonstrated improved

Hepatocyte proliferation POD 2

Hepatocyte proliferation POD 5
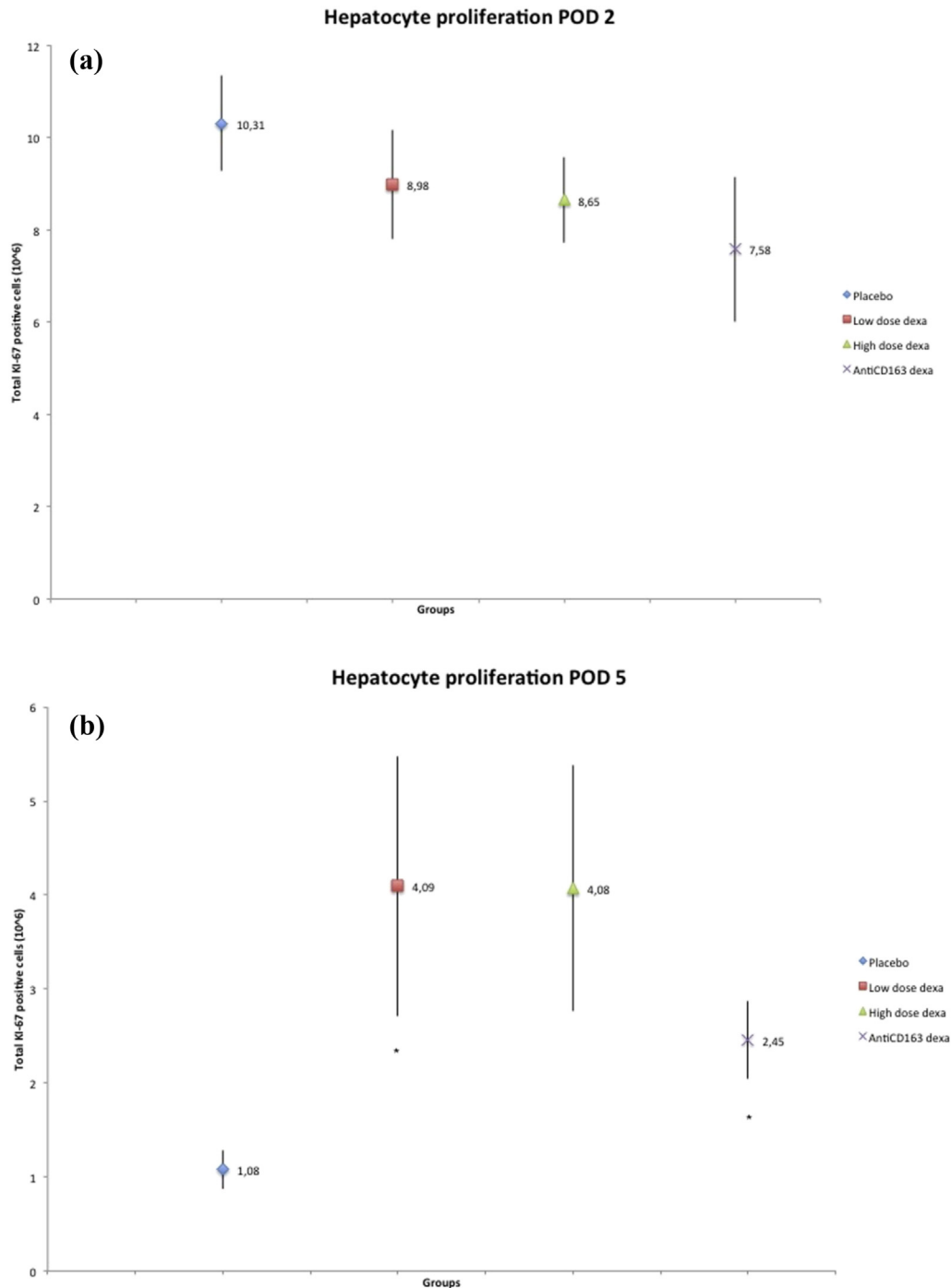
Fig. 5. (**a**+**b**). **Hepatocyte proliferation.** The hepatocyte proliferation on POD 2 and POD 5. Mean (±SE). Significant differences have been indicated with symbols. POD 5: Placebo vs. LDD (*p = 0.015) and placebo vs. anti-CD163-dex (**p = 0.015).

survival in the group of rats undergoing suppression of cytokine production. A possible explanation for this apparent discrepancy could be that Tsutsumi el al. performed their studies in rats following a 90% liver resection, in contrast to the 70% liver resection used by Meijer et al. It is possible, that different intracellular pathways might be turned on or off, depending on the amount of liver resected. Glanemann et al. investigated the effect of preoperative administered systemic methylprednisolone (MP) in partially hepatectomised rats [39]. They showed that MP tended to be associated with lower liver weights, but had no apparent impact

on the remnant liver to body weight ratio [39], results that are similar to our findings with HDD in the present study. With regard to proliferative activity, Glanemann et al. found no significant differences comparing MP and placebo treated rats, as assessed by mitotic index, by percentage of Ki-67 positively immunostained hepatocytes, and by cyclin-D1 expression [39]. In this regard, their results are in agreement with our findings in the present study. A common feature of the experimental strategy in the studies discussed here, that investigated proliferation, was that this was assessed by semi-quantitative methods [37–39]. As discussed later,

Table 1
Biochemistry and cytokines.

Liver biochemistry	POD 2				POD 5			
	Placebo	LDD	HDD	AD	Placebo	LDD	HDD	AD
ALT (U/l)	127.74 (13.46)	122.59 (12.65)	127.86 (11.69)	111.89 (15.52)	32.40 (4.71)	34.54 (4.23)	37.00 (4.61)	49.68 (5.35) *p 0.038
AST (U/l)	254.93 (19.28)	256.49 (28.10)	243.83 (26.99)	211.91 (30.34)	84.59 (4.94)	85.78 (5.71)	105.45 (11.43)	145.95 (32.00)
ALP (U/l)	496.25 (18.48)	498.88 (51.29)	401.00 (25.30) #p 0.042	498.75 (28.88)	229.50 (9.46)	230.63 (20.79)	250.25 (23.74)	218.25 (10.51)
Albumin (g/l)	9.90 (0.48)	9.63 (0.30)	10.48 (0.42)	9.59 (0.62)	9.62 (0.95)	10.31 (1.09)	9.33 (0.45)	11.24 (0.57)**p 0.020
Bilirubin (μmol/l)	4.61 (0.65)	4.61 (0.36)	4.51 (0.94)	4.38 (0.54)	0.85 (0.16)	0.88 (0.09)	1.05 (0.24)	1.08 (0.09)
Haptoglobin (g/l)	0.262 (0.029)	0.254 (0.046)	0.303 (0.030)	0.299 (0.043)	0.572 (0.075)	0.519 (0.079)	0.649 (0.039)	0.386 (0.040)**p 0.020
Cytokine profile	POD 2				POD 5			
	Placebo	LDD	HDD	AD	Placebo	LDD	HDD	AD
TNF-α (pg/ml)	6.56 (2.23)	5.53 (1.33)	8.87 (4.96)	29.30 (25.26)	4.71 (1.76)	1.37 (1.05)	4.37 (2.15)	1.37 (0.63)
α2-macro- globulin (μg/ml)	583.37 (150.53)	617.49 (107.62)	744.89 (202.24)	741.49 (164.76)	821.82 (363−28)	841.78 (353.71)	983.51 (358.69)	155.63 (24.11) *p 0.038**p 0.028
IL-6 (pg/ml)	79.57 (41.34)	126.28 (56.07)	365.27 (300.67)	600.38 (484 21)	37.25 (4.95)	11.33 (9.87)	49.72 (31.50)	1.9 (0.86)**p 0 0.38

The liver biochemistry and cytokine profile. Mean (SE). There are significant differences between the anti-CD163-dex (AD) group, and the placebo (*) and HDD (**) group. There is a significant difference between the HDD group and placebo (#).

this strategy is associated with several potential pitfalls [33].

During hepatic resection, it is important to reduce blood loss that requires transfusion, since this is known to have a negative impact on postoperative morbidity and mortality [8,40]. Vascular occlusion, for example by Pringles maneuver, is a frequently used method to minimize blood loss [41]. However, this causes IR injuries to the liver [9]. In a previous study we showed that anti-CD163-dex treatment in rats protected against development of apoptosis after IR injuries [23]. Thus we can speculate, on the basis of our present results, that anti-CD163-dex could reduce IR injuries without influencing liver regeneration and may, therefore, be a good candidate for pharmacological preconditioning.

Body weight loss is a strong marker of general distress in animals [42]. In this study, rats in the HDD group had a significant fall in body weight throughout the study. On the other hand, anti-CD163-dex treated rats showed an increase in body weight comparable with placebo, and had fewer complications in terms of infections than HDD treated animals. In a study by Graversen et al., anti-CD163-dex as an ADC had no major systemic effects as measured by reduction in thymus, spleen, or total body weight, whereas this was observed after systemic HDD [22]. This suggests that by targeting dexamethasone to macrophages, one may be able to avoid the side effects seen with systemic administration, and that the effect is also observed using targeted liposomes loaded with dexamethasone.

ALT has been widely used as a biochemical marker of hepatic injury [43]. POD 2 ALT levels were lowest in the anti-CD163-dex group, although there were no significant differences between groups. However, ALT was significantly higher in the anti-CD163-dex group compared with placebo on POD 5. As ALT has a half-life around 47 h, the surgical stress to the liver is expected to result in elevations in ALT levels primarily within the first two days [26,43]. The unexpected results found in the present study may, of course, have occurred by chance. An alternative explanation could be that anti-CD163-dex treatment resulted in increased damage to hepatocytes. However, this conclusion would contrast with the findings of a separate study by our group, in which we were unable to demonstrate a protective effect of anti-CD163-dex treatment on hepatocyte apoptosis after IR injury [23]. A third alternative explanation could be that anti-CD163-dex treatment delays apoptosis in many hepatocytes injured by surgery, since levels were lower (although this did not reach significance) in this group on POD 2.

Albumin is an established marker of the hepatic ability to synthesize proteins [43]. In the present study, the anti-CD163-dex group had significantly higher albumin levels compared with the HDD group. Glanemann et al. also showed that rats treated with systemic MP had significantly reduced albumin levels [39]. This underlines the difference between systemic and macrophage targeted corticosteroids, i.e. systemic treatment impairs albumin synthesis by the liver.

The inflammatory response to surgical stress is known to cause a rise in acute phase proteins such as haptoglobin and α2M [26,44]. Blood levels of haptoglobin and α2M were significantly lower in the anti-CD163-dex group compared with HDD, consistent with a reduced inflammatory response in the liver. A postoperative rise in IL-6 has been associated with IR injuries and adverse patient outcome [13,45]. In our study, anti-CD163-dex significantly reduced levels of IL-6 compared with HDD. The fact that anti-CD163-dex attenuated the cytokine response indicates its anti-inflammatory potency and potential protection against IR injuries. Both in a rat and a porcine LPS-model, anti-CD163-dex has been shown to have an anti-inflammatory effect in terms of reduced cytokines, comparable with fifty times higher concentrations of free dexamethasone [22,46].

The specific expression of CD163 in macrophages makes it an interesting therapeutic target, opening up the possibility of directing a specific effect restricted to localized sites of inflammation such as are seen following PHx [47]. The anti-inflammatory action of anti-CD163-dex may also be of interest in the future treatment of toxic and autoimmune liver disorders such as AHH, primary biliary cirrhosis, autoimmune hepatitis, and primary sclerosing cholangitis. By using a locally active treatment, it may be possible to reduce or abolish the complications seen after long-term therapy with high-dose corticosteroids. However, these possibilities must be the subject for future research.

A strength of the present study is our use of design-based stereological methods in the histological evaluation. Unlike the semi-quantitative methods used in most other studies, stereology makes use of randomization to ensure that the analyzed part of the tissue is representative of the whole structure [33]. This allows quantitative data to be collected in an unbiased way, reducing the subjectivity that is an integral part of non-stereological techniques. Furthermore, it takes tissue shrinkage into account in an unbiased fashion [33,36]. A limitation of the study might be the relatively small number of animals in each group (n = 8). A small number gives a risk of overlooking an actual difference between the groups.

5. Conclusion

In conclusion, the present study shows that treatment with anti-CD163-dex does not adversely affect regeneration of the liver after PHx in rats, although it reduces the inflammatory response judged by inflammatory markers.

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Ethical Approval

No Ethical Approval necessary.

All animal experiments were performed under the approval of Danish Animal Experiment Inspectorate, Copenhagen, Denmark (license number 2012-15-2934-00591 expansion), and in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institute of Health, USA [24].

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Author contribution

• Study design: Betina Norman Jepsen, Kasper Jarlhelt Andersen, Anders Riegels Knudsen, Jonas Heilskov Graversen and Frank Viborg Mortensen.
• Data collection: Betina Norman Jepsen.
• Data analysis and interpretation: Betina Norman Jepsen, Kasper Jarlhelt Andersen, Anders Riegels Knudsen, and Frank Viborg Mortensen.
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Conflicts of interest

Holger Jon Møller, Søren Kragh Moestrup and Jonas Heilskov Graversen own shares in Affinicon, which holds IP protecting the use of CD163 drug targeting.

Trial registry number

Not an RCT.

Guarantor

Kasper Jarlhelt Andersen.
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