MAJOR ARTICLE

Liver-Stage Development of *Plasmodium falciparum*, in a Humanized Mouse Model

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Background. The liver stage of the human malaria parasite *Plasmodium falciparum* is the least known, yet it holds the greatest promise for the induction of sterile immunity and the development of novel drugs. Progress has been severely limited by the lack of adequate in vitro and in vivo models.

Methods. Recently, it was found that immunodeficient mice transgenic for the urokinase plasminogen activator allow survival of differentiated human hepatocytes. We confirm this finding but show that hepatocyte survival is short lived unless nonadaptive defenses are simultaneously depleted.

Results. By controlling macrophages and NK cells, we readily effected the long-term secretion of human serum albumin and human α-1 antitrypsin in mouse serum (at 3 months, the proportion of repopulated mice increased from 0% to 60% and from 22% to 80%, respectively; $P < .0001$). *P. falciparum* sporozoites delivered intravenously into mice readily infected transplanted human hepatocytes and developed into liver schizonts. Their size was twice as large as what was seen in vitro and was comparable to that found in humans and chimpanzees.

Conclusion. These results emphasize the importance of nonadaptive defenses against xenotransplantation and lead to development of small laboratory models that, because they can harbor human hepatocytes, provide novel opportunities to study intrahepatic pathogens, such as those causing malaria and hepatitis.

The intrahepatic phase of development of *Plasmodium*-malaria parasites precedes the pathogenic intraerythrocytic phase and is by far the most difficult to study—and, therefore, is the least characterized of these parasites' life-cycle stages. The intrahepatic phase is, however, of considerable interest from the point of view of vaccine development, because it is the only stage against which sterile immunity has been induced in humans by vaccination [1, 2]. From a therapeutic point of view, the intrahepatic phase is biochemically distinct from other stages [3], and therefore the study of hepatic stages is of considerable fundamental and practical value. However, such study has been limited by practical and ethical constraints on the use of biological material, even though the latter is critical for research on hepatitis and malaria, each of which kills several million individuals each year [4, 5].

Moreover, primary hepatocytes in vitro do not replicate or retain their differentiated functions; and, in vivo, only human cells or those of higher primates such as the chimpanzee are receptive to *P. falciparum* and susceptible to infection with hepatitis B or C virus [6–8]. There have therefore been many attempts to develop laboratory models supporting the survival of human hepatocytes; but these models have met with limited

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success [9–11]: on the one hand, transplantation of human hepatocytes into immunodeficient mice has been found to improve the outcome, although the viability of the hepatocytes has been found to be low and variable [9–11]; on the other hand, the possibility that there are genetic defects, such as those relating to albumin–urokinase-type plasminogen activator (Alb-uPA), that might confer a growth advantage to transplanted nontransgenic hepatocytes was identified long ago [12]. However, it is the combination of both types of genetic mutation—that is, a mutation such as that to Alb-uPA, which facilitates survival of human hepatocytes, and a mutation such as that to either SCID or recombination-activating gene (RAG)–2, which results in immunodeficiency—that led to a major breakthrough, which was based on transplanted human hepatocytes' receptivity to human hepatotropic viruses [13–17]. The survival of lentivirally transduced primary human hepatocytes [18] and of embryonic mouse cell lines [19] in uPA-SCID mice also favors the hypothesis that it is the combination of both mutations that most fosters hepatocyte growth.

On the other hand, we have recently drawn attention to the importance of nonadaptive defenses: using erythrocytic forms of *P. falciparum,* we found that this parasite survived in human red blood cells in SCID mice only when both blood and tissue macrophages were simultaneously depleted—or when their parasite-dependent proliferation was at least controlled [20].

Therefore, on the basis of these combined findings, we performed further studies with human hepatocytes in the uPA-SCID model, studies in which nonadaptive immune defenses were depleted by a pharmacological treatment. We assessed the results in terms of the survival and the development of liver-stage *P. falciparum* in human hepatocytes transplanted into immunodeficient mice.

MATERIALS AND METHODS

Animals. Homozygous Alb-uPA mice from Jackson Laboratories and homozygous SCID mice from IFFA-CREDO were maintained under pathogen–free conditions.

To generate uPA-SCID mice, we crossed Alb-uPA and SCID mouse lines, and, through selective backcrosses ($n = 6$), bred the uPA-SCID trait to zygosity. The uPA trait was identified by polymerase chain reaction (PCR) performed on mouse-tail DNA. The chimeric Alb-uPA transgene corresponds to the albumin promoter, the murine uPA gene, and part of the human growth-hormone gene [21]. To differentiate the transgene-derived sequence, we used the following primer pair: p1 (5′-ATTCTGGAGGACCGCTTATCTGT-3′), located within uPA sequence; and p2 (5′-CTTGAACCCAGGAGGCGGAGATT-3′), located within the human-growth-hormone sequence. PCR conditions were as follows: 40 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

Homozygosity for the SCID trait was analyzed by quantification of total serum IgG, by use of a sandwich ELISA. Mice with total IgG concentrations of 10–30 μg/mL, determined according to the recommendations of the supplier (Charles River Laboratories), were considered to be homozygous for the SCID trait. In the present study, only mice heterozygous for the uPA transgene were used for the transplantation experiments.

Isolation and transplantation of human hepatocytes. Primary human hepatocytes were isolated from surgically collected liver-biopsy samples, in accordance with French national ethical regulations (article L-1245-2 of the Huriet laws) and by use of Collagenase H (Roche Molecular Biochemicals) perfusion and were separated from nonparenchymatous cells by Percoll fractionation, as described elsewhere [22]. Viable cells were identified by trypan-blue exclusion.

For liver transplantation, $\sim 5 \times 10^5$–6×10^5 human hepatocytes were injected into the spleens of 10–14-day-old mice [18]. After transplantation, half of the mice were treated, and half remained untreated. Serum and liver-biopsy samples were obtained at 4, 8, and 12 weeks after transplantation. At 4-day intervals beginning 2 days after transplantation, mice in the treated group were injected intraperitoneally with liposome-encapsulated clodronate (50 μL of a 50%-hematocrit liposome solution) [23]. For depletion of NK cells, mice received 1 mg of purified anti–thrombomodulin β-1 (anti-TM β-1) mouse monoclonal antibody (MAb) (specifically, anti–mouse interleukin-2R β chain) at monthly intervals beginning 2 days after transplantation [24]. Both of these agents have well-documented effects. Clodronate depletes, for >1 week [24], >90% of blood and tissue macrophages after the first liposome injection; however, in the present study it was injected every 4 days, because such a regimen had previously been found to be necessary in mice that had received human transplants [20]. The effect of anti–TM β-1 MAb also has been documented: a monthly injection has been shown to prevent NK-cell recovery [24]. In addition, in preliminary experiments, batches of clodronate and batches of anti-NK antibodies were assessed for efficacy. In normal BALB/cJ mice, fluorescence-activated flow sorter (FACS) analysis performed 2 days after liposome injection showed that batches of clodronate depleted ~90% of blood cells that were positive for the F4/80 marker. FACS analysis of $DX5^+CD3^-$ spleen cells from antibody-treated BALB/cJ mice, using anti-DX5 and anti-CD3 MAbs (clone 17A2) to assess the efficacy of each batch of anti–NK-cell MAbs, revealed that >80% of such cells had been depleted.

ELISA of human serum albumin (HSA) and human α-1 antitrypsin (α1AT). The levels of HSA and human α1AT in serum from SCID mice were quantified by ELISA. Mouse anti–HSA (0.1 μg/mL, clone HSA-9; Sigma) and goat anti–human α1AT (11 μg/mL; Rockland) were used as capture antibodies for coating and were blocked with 1% bovine serum albumin.

Mouse serum diluted 1:10 (75 μL/well) was incubated at 4°C overnight. Horseradish peroxidase (HRP)–conjugated rabbit anti–HSA (0.16 μg/mL; Sigma) and HRP-conjugated rabbit anti–human α1AT (2 μg/mL) were used as secondary antibodies. Concentrations (in micrograms per milliliter) were determined on the basis of comparison with the standard curve for purified HSA and human α1AT (Sigma). The ELISA result was considered to be positive if the optical density (405 nm) values were greater than the mean (±2 SD) values for 14 nontransplanted uPA-SCID mice. Qualitative comparisons were performed by use of the χ^2 test or the unpaired *t* test. $P < .05$ was considered to be statistically significant.

Reverse-transcription (RT)–PCR. For detection of HSA, total RNA was isolated from chimeric human-mouse liver, mouse liver, and human liver, by use of the RNeasy Protect Mini kit (Qiagen). Equal amounts of RNA from each sample were subjected to cDNA synthesis using random primers. HSA-specific primers—5′-CATTAGCTGCTGATTTTGTTGAAAG-3′ (sense) and 5′-TGTGCAGCATTTTGTGACTCTG-3′ (antisense)—were used to detect HSA transcripts (523-bp band). PCR conditions were as follows: initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72° C for 5 min. Samples (20 μL) of PCR product were analyzed by electrophoresis using 2% agarose gel and ethidium bromide.

For detection of parasite transcripts, total RNA was extracted, as described above, from (1) transplanted and nontransplanted infected mouse-liver tissue samples collected 5 days after inoculation with sporozoites, (2) uninfected human liver tissue, and (3) *P. falciparum* erythrocytic forms. RT was performed with a final volume of 20 μL and used 4 μg of total RNA and 200 U of M-MLV Reverse Transcriptase (Invitrogen) with specific primers LSA1-R and HSP70-R (from the *lsa1* and *hsp70-1* genes, respectively, of *P. falciparum*). Nested RT-PCR was performed with 2 μL of RT-reaction product and used 125 μmol/L of each dNTP, 125 nmol/L of each primer, 2 U of *Taq* polymerase (Qiagen), and the following primers: for liver-stage antigen 1 (LSA1), LSA1-F (5′-AATCTAACTTGAGAAGTGG-3′) and LSA1-R (5′-CTGCATCGTCATTTATTATG-3′), then LSA1-NF (5′-TCTTCAAATTCTTGGAATCG-3′ and LSA1-NR (5′-GTTCAATTAATTTCCCTTCC-3′) (band of 252 bp); for *P. falciparum* heat-shock protein 1 (*Pf*HSP70-1), HSP70-F (5′-AGGTATAGAAACTGTGGGTGG-3′) and HSP70-R (5′-GATTGGTTGGCATACAGCTTC-3′), then HSP70-NF (5′-GAAATACTGTCATCCCAACC-3′) and HSP70-NR (5′-GCTTCAGAATCAGCATTCGAG-3′) (529-bp band). Direct PCR was performed as a control, to rule out genomic DNA contamination of RNA preparations.

Immunohistological staining. Mouse-liver–biopsy samples were fixed in 10% formalin and were embedded in paraffin. For detection of transplanted human hepatocytes, 5-μm sections pretreated with Dako Biotin Blocking System (Dako) were immunostained with an MAb against human hepatocytes (Clone OCH1E5, 1:20 dilution; Dako), by use of the Dako ARK system. Similarly, 5-μm sections were immunostained with rabbit anti–human α1AT antibodies (Dako), diluted to 1:2000, at room temperature for 1 h. Slides were developed by use of a secondary anti-rabbit antibody conjugated with HRP (EN-VISION-kit; Dako). For detection of macrophages, sections were immunostained with rat MAb Mac2, in culture supernatant (TIB 166; American Type Culture Collection) diluted to 1:4, at room temperature for 1 h. HRP-labeled anti-rat antibodies (1:500 dilution) were incubated for 30 min and were revealed by use of diaminobenzidine. Endoperoxidase activity was blocked by first treating the slides with 0.3% hydrogen peroxide in PBS for 5 min at room temperature. Sections were counterstained with Meyer's hematoxylin (Dako).

Detection of parasites was performed 5 days after the samples had been infected with *P. falciparum* sporozoites. Indirect fluorescent-antibody testing was performed on serial sections (5 μm) of liver tissue after they had been stained with hematoxylin-eosin. Polyclonal anti-*Pf*HSP70-1 antibodies obtained from immunized mice [25], anti-LSA1 antibodies obtained from immunized chimpanzees [26, 27], and anti-circumsporozoite (CS)–protein mouse MAb 2A10 (CS/MAb 2A10) [28], were used to specifically detect liver-stage *P. falciparum,* and (1) human affinity–purified antibody against merozoite surface protein 3 (MSP3) [29], (2) serum from a malaria-naive individual, and (3) serum from a malaria-naive mouse were used as negative controls. All serum samples were diluted to 1:100, except for CS/MAb 2A10, which was diluted to 1:500. Secondary, Alexa 488-labeled anti-mouse or anti-human antibodies (Molecular Probes) were diluted to 1:600.

Quantitative imaging. Quantitative imaging was performed on liver-biopsy samples obtained , at 1 and 3 months after transplantation, from 3 untreated and 3 treated mice; 3 sections from each mouse were immunostained with rabbit anti–human α1AT. Adobe PhotoShop software (version 6.02) was then used to identify and count the pixels corresponding to either the total surface of each of the 3 sections from each mouse (by use of Meyer's hematoxylin staining) or human-hepatocyte clusters (as determined on the basis of anti–human α1AT reactivity). The percentage of liver repopulation by transplanted human hepatocytes was expressed as the cell surface that stained positive for human α1AT divided by the total cell surface of the tissue section. Qualitative comparisons were performed by use of Fisher's exact test. $P < .05$ was considered to be statistically significant.

P. falciparum *infections.* *P. falciparum* sporozoites (strain NF54) were obtained from the salivary glands of infected mosquitoes *(Anopheles stephensi)* 14–16 days after the insects had been membrane fed NF54 cultured gametocytes; $\sim 5 \times 10^5$–

6×10^5 sporozoites were intravenously (iv) injected into mice 1–2 months after transplantation. Nontransplanted or transplanted mice negative for HSA were used as negative controls.

RESULTS

Increased survival of transplanted human hepatocytes, by control of mouse nonadaptive immune defenses. Human hepatocytes transplanted into untreated uPA-SCID mice produced HSA only transiently during the first month after transplantation, and it became undetectable 3 months after transplantation (figure 1*A* and 1*B*). Immunohistochemical analysis of liver sections from mice 6 weeks after transplantation showed that intrasplenically injected hepatocytes indeed had migrated to the liver parenchyma, where they formed clusters labeled by a human-hepatocyte marker (figure 2*A*) and by anti–human α1AT (data not shown). However, they were also surrounded by numerous mononuclear cells, primarily macrophages (labeled by Mac2 MAb in figure 2*B*). At 3 months after transplantation, immunohistochemical investigations of serial sections of liver revealed that human-hepatocyte clusters were no longer labeled by anti–human α1AT—that is, they no longer produced this human protein (figure 2*C*)—and were surrounded by a denser ring of numerous macrophages (figure 2*D*); this result indicates that transplanted human hepatocytes were no longer functional and is in agreement with the finding that HSA was no longer detectable in the serum samples of mice into which human hepatocytes had been transplanted (figure 1*A*).

These in situ observations showing the recruitment of cells from the nonadaptive immune system are reminiscent of our previous attempts to transplant *P. falciparum*–infected human erythrocytes into SCID mice: transplants were successful only in macrophage-depleted SCID mice treated with liposome-encapsulated clodronate, a toxin actively ingested by macrophages [20, 30]. We therefore reason that the combination of a genetic

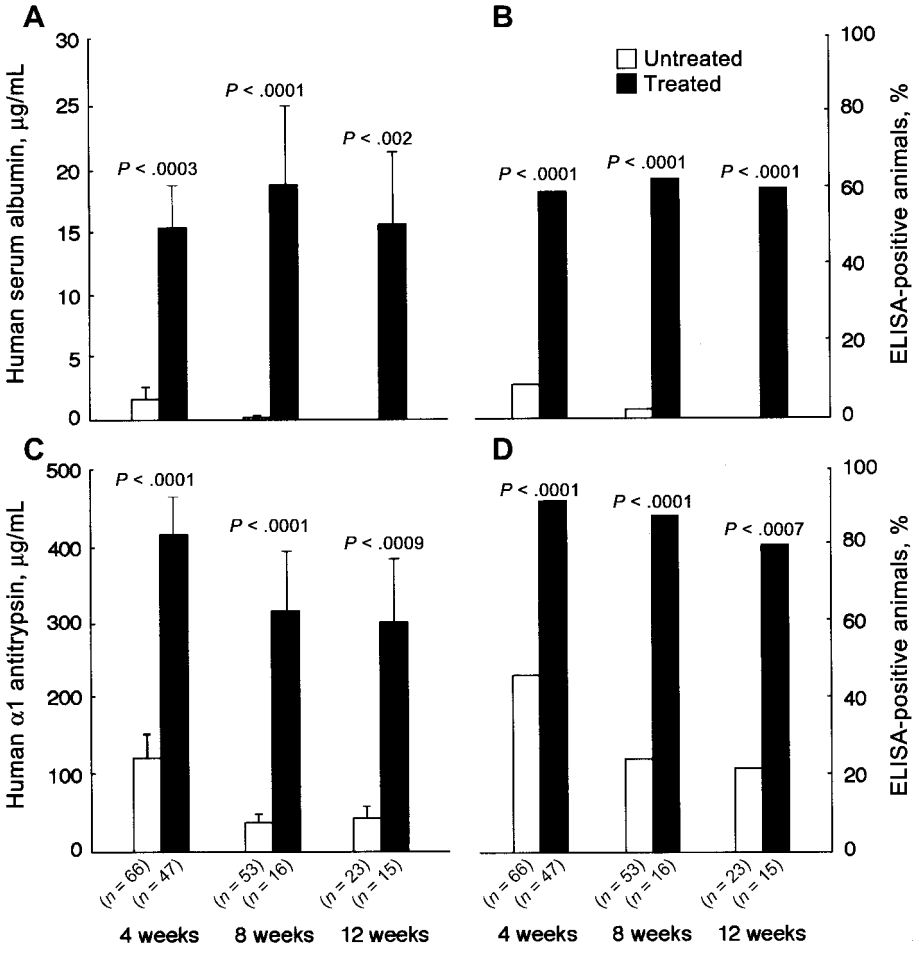

Figure 1. Secretion of human serum albumin and human α-1 antitrypsin, in treated and untreated uPA-SCID mice into which human hepatocytes have been transplanted. The serum levels of human serum albumin *(A)* and human α-1 antitrypsin *(C)* were determined by use of standard sandwich ELISA. The results are expressed as the percentage of mice positive for expression of human serum albumin *(B)* and human α-1 antitrypsin *(D)*, compared with control mice (see Materials and Methods). Statistical analysis was performed by use of the χ^2 test or unpaired *t* test. The number of mice investigated, for each group and each period, is shown in parentheses.

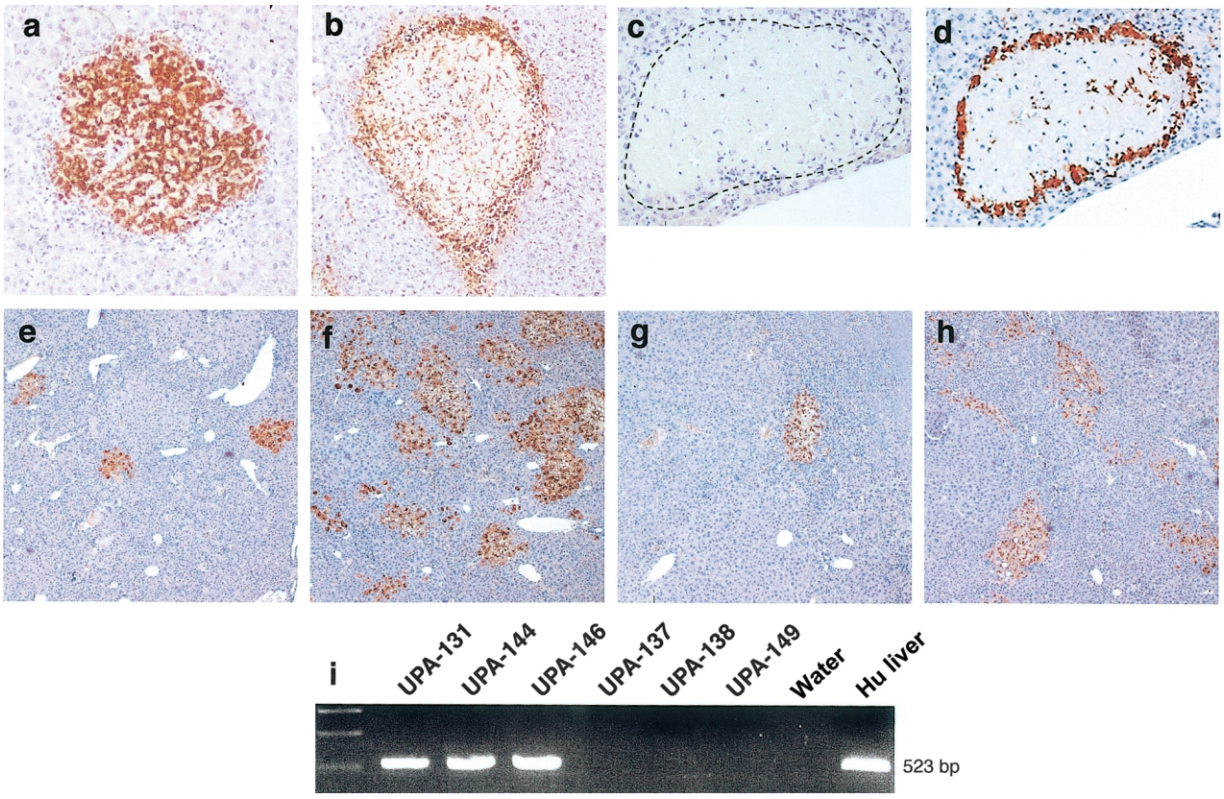

Figure 2. Immunohistological analysis of human hepatocytes transplanted into mouse liver. Chimeric human-mouse liver sections were analyzed after being labeled with various antibodies (revealed by the substrate of peroxidase *[brown]*). Six weeks after human hepatocytes had been transplanted into untreated mice, serial sections of liver were immunostained with either monoclonal antibody against human hepatocytes or anti-mouse macrophage monoclonal antibody Mac2; 12 weeks after transplantation, serial sections of liver *(C* and *D)* from an untreated mouse were immunostained with either anti–human α-1 antitrypsin antibody *(C)* or anti-mouse macrophage monoclonal antibody Mac2 *(D).* Serial sections of liver from untreated *(E* and *G)* and treated *(F* and *H)* mice were immunostained with anti–human α-1 antitrypsin antibody, 1 month *(E* and *F)* and 3 months *(G* and *H)* after transplantation. Reverse-transcription polymerase chain reaction was performed on transcripts of human serum albumin in liver samples from treated (uPA-131, uPA-144, and uPA-146) and untreated (uPA-137, uPA-138, and uPA-149) mice, 3 months after transplantation *(I);* human liver (Hu liver) and water were used as positive and negative controls, respectively.

lymphocyte deficiency and agents that are able to reduce nonadaptive defenses should be beneficial to transplantation.

After transplantation of human hepatocytes, half of each group of transplanted Alb-uPA/SCID mice was treated with clodronate encapsulated in liposomes that target tissue macrophages [23]; because NK cells have been reported to be involved in xenotransplant rejection, an anti-NK antibody was used simultaneously [31, 32]. The other half of each group of transplanted mice, which received human hepatocytes from the same donors, were not treated and were used as negative controls. As is shown in figure 1, during the first 3 months after transplantation both (1) the proportions of mice secreting HSA and human α1AT and (2) the amounts of HSA and human α1AT secreted were markedly increased by this procedure. Indeed, both the amounts of HSA and human α1AT secreted by treated mice (figure 1*A* and 1*C*) and the proportion of mice secreting HSA and human α1AT (figure 1*B* and 1*D*) were markedly higher 1 month after transplantation and remained unchanged during the subsequent 2 months; in contrast, among the untreated control mice, both the proportions of mice secreting HSA and/or human α1AT (figure 1*B* and 1*D*) and the amounts HSA and human α1AT secreted (figure 1*A* and 1*C*) decreased during the first 3 months after transplantation.

Immunohistochemical analysis confirmed our initial hypothesis that a strong reduction in the numbers of macrophages and NK cells in treated mice was associated with an increase in both the number of human-hepatocyte clusters and the number of hepatocytes per cluster. At both 1 month (figure 2*E* and 2*F*) and 3 months (figure 2*G* and 2*H*) after transplantation, there was a visible decrease in the number of myeloid cells and an increase in the number of human-hepatocyte clusters' surfaces. This improvement was quantified by measuring, in liver sections from transplanted mice, the percentage of cell surface that stained positive for human α1AT. A significantly higher percentage of human-hepatocyte repopulation was observed in treated mice, compared with untreated mice (human-hepato-

cyte surface, 17.7% ± 2.1% vs. 3.1% ± 1.5%, respectively [$P = .0006$], at 1 month and 26.9% ± 4.2% vs. 6.5% ± 2.4%, respectively [$P = .002$], at 3 months). In agreement with these results is the finding that RT-PCR performed 3 months after transplantation detected HSA transcription in RNA samples from chimeric human-mouse liver specimens from treated mice but not in those from untreated mice (figure 2*I*). These results indicate that innate immune mechanisms influence the survival and functionality of human hepatocytes transplanted into mouse liver.

Intrahepatic development of* P. falciparum *in transplanted human hepatocytes. Sporozoites, the invasive form of *P. falciparum* present in the mosquito, were injected iv into transplanted and control mice, and liver-biopsy samples were collected 5 days later (in humans, maturation of *P. falciparum* requires 5–6 days). A total of 14 mice into which human hepatocytes from 4 different donors had been transplanted were injected with sporozoites from 4 batches of mosquitoes 1–2 months after transplantation. *P. falciparum* liver forms, 50–60 μm in diameter, were readily detected, by light microscopy, in hematoxylin-eosin–stained sections (figure 3*A*). Indeed, the density of the parasites in liver sections was similar to that observed in higher primates [6] and apparently related to HSA levels—that is, the largest numbers of parasites were seen in mice having the highest HSA levels, ~100 μg/mL, and only HSA-positive mice were receptive to development of parasites.

Morphological indications were confirmed by immunofluorescence labeling by use of parasite-specific reagents and RT-PCR. Liver forms in tissue sections from the different groups of mice were specifically labeled by use of (1) MAb specific to the CS protein, a molecule expressed in sporozoites and carried over into the liver stage of *P. falciparum* [3] (figure 3*B1*), and (2) antibody against HSP70-1 (figure 3*B2*), a parasite heat-shock protein expressed at high levels in multinucleated liver schizonts but not expressed in either sporozoites or liver trophozoites of *P. falciparum* [25]. Antibody specific to LSA1, an antigen expressed solely during the liver stage [26, 27], also reacted specifically with liver forms (figure 3*B3*). In contrast, control antibodies, which were directed against either MSP3, an antigen expressed only during the blood stage (and not in mature liver forms), or nonmalarial antigens yielded negative results on serial sections of liver from positive mice (figure 3*B4*); conversely, liver form–specific antibodies were not present in liver sections (>100 screened) from nontransplanted infected control mice.

These results were confirmed by RT-PCR using primers specific for parasite and human genes. At 5 days after inoculation, HSA transcripts were detected both in 7 of 7 HSA-secreting mice and in 3 of 7 transplanted mice without detectable HSA in their serum but not in 3 nontransplanted control mice (figure 3*E*). RT-PCR using parasite-specific primers derived from LSA1 sequences and from HSP70 sequences yielded homogeneous results (figure 3*C–E*), in agreement with the findings discussed above. Corresponding parasite mRNA was detected in 7 of 10 mice in which HSA transcripts were detected (figure 3*C* and 3*D*) but not in 4 human-hepatocyte–transplanted mice that were negative for HSA (figure 3, uPA-1). Hence, morphological observations, antigens, and gene expression indicated that the intrahepatic parasites obtained had the features of maturing *P. falciparum* liver schizonts that develop only in differentiated hepatocytes [6, 33, 34]. However, the final stage of schizogony, described in vivo as aposchizogony [35, 36], was not observed at day 5 after transplantation, because it occurs during the latest phase of liver-stage maturation of *P. falciparum.*

DISCUSSION

In the present study, we have shown that the invasion by and development of *P. falciparum* can be demonstrated in a small laboratory model of human-hepatocyte transplantation. These results corroborate the value of combining (1) the use of animals with a genetic defect in adaptive immunity and (2) the control of nonadaptive defenses, as a way to improve the results of xenotransplantation of human hepatocytes into mice. These results have both conceptual and practical implications: at the conceptual level, it confirms what also has been indicated by erythrocytic transplantation—that is, that it is possible to more efficiently break the species barrier by impairing both types of immunity; at the practical level, it opens novel areas of research in malaria.

Our approach relied on combining (1) the use of mice with 2 genetic deficiencies, affecting hepatocytes and T and B lymphocytes, and (2) a pharmacological treatment aimed at control of nonadaptive defenses. The latter constitutes a very strong line of defense against foreign organisms, pathogens, or xenotransplantation [37, 38]. In nonimmune individuals, nonadaptive defenses very efficiently contribute to the elimination of foreign tissues such as transplants or cancer cells [39, 40] and to the destruction of large proportions of invading microorganisms, such as parasites (e.g., 99.9% of blood-stage *P. falciparum* [41]) or bacteria [42]. Conversely, the nonspecific stimulation of phagocytes by, for example, bacille Calmette-Guérin or *C. parvum* can by itself ensure the complete clearance of many pathogens [43, 44] and is currently used in the treatment of cancers [45, 46]. The very significant improvement in transplantation results that was observed in the present study corroborates the importance of these defenses, and, in addition, the reduction of NK cells could have contributed either directly [32] or indirectly, via NK cells' contribution to macrophages activation [47, 48].

The Alb-uPA transgenic mouse expresses a transgene that leads to the death of transgene-carrying hepatocytes but not to the death of transplanted hepatocytes [12, 49]. The develop-

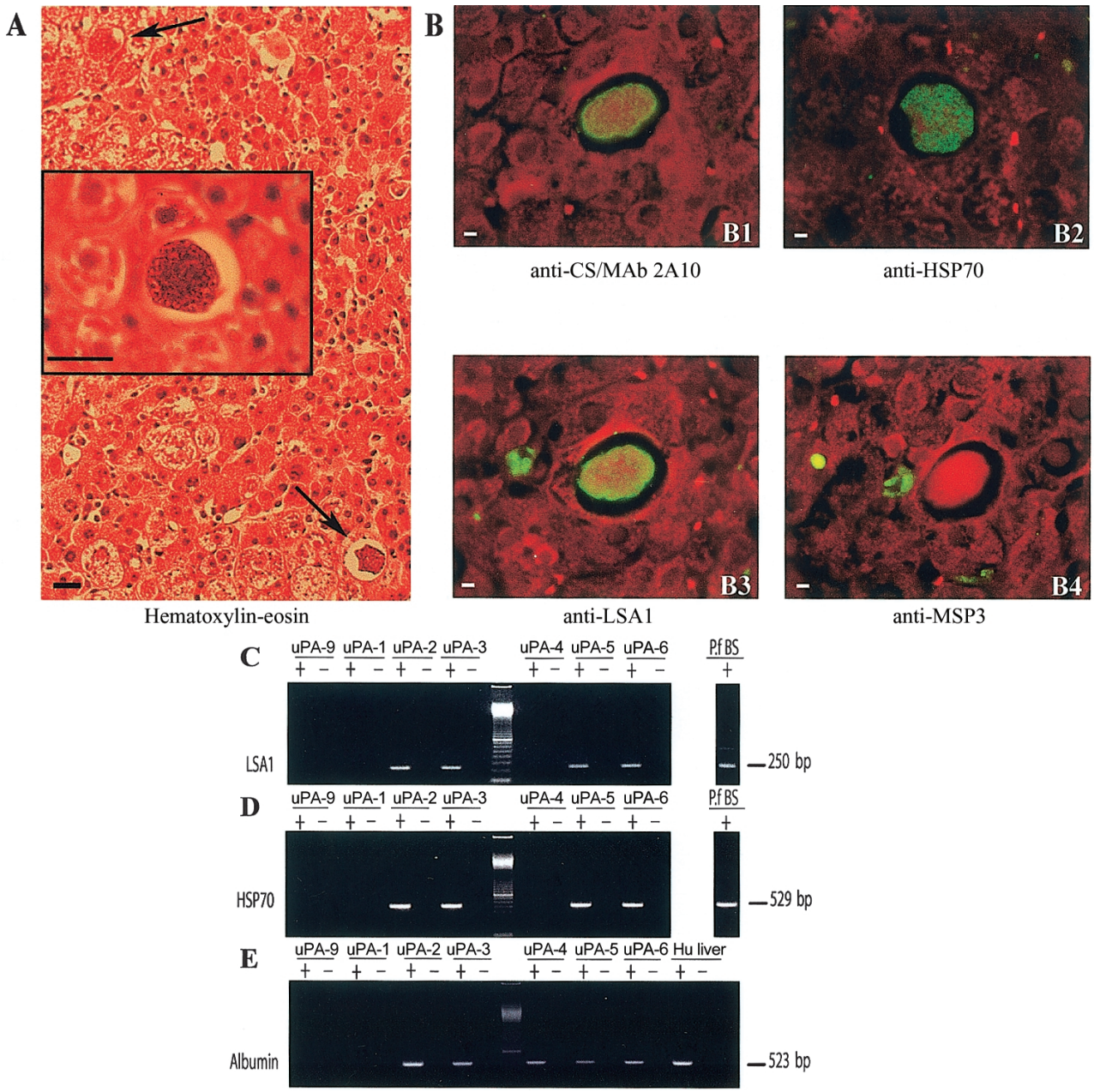

Figure 3. Liver-stage development of *Plasmodium falciparum* in chimeric human-mouse liver. Sections of liver were stained with hematoxylin-eosin *(A,* showing 2 developing *P. falciparum* liver schizonts 5 days after infection *[arrows]* and a third schizont *[inset]* from the same mouse; bars, 50 μm). *P. falciparum* liver forms were labeled by use of various antibodies in an immunofluorescent-antibody test *(B* bars, 10 μm): anti–circumsporozoite-protein mouse monoclonal antibody 2A10 (anti-CS/MAb 2A10) *(B1),* anti–heat-shock protein 70 antibody (anti-HSP70) *(B2),* anti–liver-stage antigen 1–protein antibody (anti-LSA1) *(B3),* and, as a negative control, anti–merozoite surface protein 3 antibody (anti-MSP3) *(B4).* Reverse-transcription polymerase chain reaction using LSA1 primers *(C),* HSP70 primers *(D)* was performed on liver-biopsy samples from transplanted mice (uPA1–uPA6) and a nontransplanted mouse (uPA-9), all infected with *P. falciparum* sporozoites; as a positive control, polymerase chain reaction was performed on genomic DNA from blood-stage *P. falciparum* (P.f BS/+). Reverse-transcription polymerase chain reaction using human-serum albumin primers was performed on liver-biopsy samples from transplanted mice (uPA1–uPA6) and from a nontransplanted mouse (uPA-9) and, as a positive control, on a sample of human liver (Hu Liver) *(E).* The initial reverse-transcription reaction was performed in the presence (+) and absence (−) of the reverse transcriptase.

ment of immunotolerant Alb-uPA mice has led to the development of a successful model for xenotransplantation of rat [50], woodchuck [51], tupaia [16], and human [13, 14, 52] hepatocytes; however, long-term survival of transplanted human hepatocytes was observed only in homozygous mice [13]. In contrast, the present study has shown that transplant survival can be improved in terms of duration, number of hepatocytes, and function of hepatocytes, as well as in terms of the proportion of

positive mice, in heterozygous mice depleted for macrophages and NK cells. Tateno et al. have recently reported that human complement secreted by transplanted hepatocytes has a significant deleterious effect on the survival of mice and, therefore, of human hepatocytes transplanted into them [52]. Therefore, it can be supposed that the combination of improvements that we have here presented, together with those recently reported, can lead to an optimized and reproducible chimeric mouse model.

Human malaria is a very sensitive marker of hepatocyte differentiation. Indeed, few of numerous hepatoma cell lines permit the development of the intrahepatic phase of *P. falciparum* [53, 54], and, thus far, none has proved able to sustain satisfactorily the complete development of the parasite. In contrast, human hepatocytes transplanted into treated uPA-SCID mice were sufficiently well differentiated to sustain the development of liver schizonts; their size, reaching 50–60 μm 5 days after inoculation with sporozoites, was as large as that which has been observed in either humans [33] or chimpanzees [6, 34] and was twice as large as that which has been observed in primary human-hepatocyte cultures in vitro [55, 56]. Furthermore, their development was very homogeneous among the liver schizonts. Given both the relatively small number of human hepatocytes transplanted, compared with the number of mouse hepatocytes, and the long journey of sporozoites from peripheral blood to the liver, it is remarkable that so many sporozoites reached a receptive human cell and developed at high densities.

The development of an in vivo model for the liver stage of *P. falciparum* provides a much-needed new tool for the study of host-parasite interactions —particularly the immune effectors of defense—and for the identification of these effectors' target antigen(s) [57, 58]. Liver-stage parasites are of particular interest because they are both at the origin of protective immunity [3, 59] and the main target of immune effectors [58]. Numerous mechanisms have been described in rodent models [3, 60], but it remains unknown which of these mechanisms may actually be effective against *P. falciparum* [61]—and, therefore, which of them would have to be preferentially induced by a vaccine [62].

Finally, the combination of this model and the model that has elsewhere been described for the blood stages of malaria [20] should ultimately provide—after the latter model has been successfully adapted, particularly with respect to the protocol of control of nonadaptive defenses, to the uPA-SCID model—access to the complete cycle of the development of *P. falciparum* in a small laboratory host, thereby providing a capability that will have numerous applications not only in the field of vaccine development but also for formal genetic studies [63, 64].

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