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ORIGINAL ARTICLE

Critical role of natural killer cells in the rejection of human hepatocytes after xenotransplantation into immunodeficient mice

Toshiyasu Kawahara,[1] Donna N. Douglas,[1] Jamie Lewis,[1] Garry Lund,[2] William Addison,[3] David Lorne Tyrrell,[2,3] Thomas A. Churchill[1,4] and Norman M. Kneteman[1,2]

1 Department of Surgery, University of Alberta, Edmonton, Alberta, Canada
2 KMT Hepatech Inc., Edmonton, Alberta, Canada
3 Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada
4 Surgical Medical Research Institute, University of Alberta, Edmonton, Alberta, Canada

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Correspondence
Norman M. Kneteman, MD, MSc, FRCSC, Department of Surgery, University of Alberta, 2D4.44 Mackenzie Center, Edmonton, Alberta T6G 2B7, Canada. Tel.: 780/407-6168; fax: 780/407-7374; e-mail: norm.kneteman@albertahealthservices.ca

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Summary

The severe combined immunodeficiency/albumin linked-urokinase type plasminogen activator (SCID/Alb-uPA) human liver chimeric mouse model has added a new dimension to studies of liver based human diseases and has important potential for study of human hepatic drug metabolism. However, it remains unclear if natural killer (NK) cell in SCID/Alb-uPA mice has an important negative impact on engraftment and expansion of human hepatocytes after transplantation. Here, we explore the role of mouse NK cells in the rejection of transplanted human hepatocytes in SCID/Alb-uPA mice. We assessed NK cell activity *in vivo*, using ^{125}I-iodo-2′-deoxyuridine incorporation assay. Low serum human alpha-1 antitrypsin (hAAT, <10 μg/ml) recipients, representing graft failure, showed resistance to engraftment of MHC class I knockout marrow (indicating high NK cell activity), while NK cell-depleted low hAAT recipients and high hAAT (>100 μg/ml) recipients accepted MHC class I knockout marrow, indicating a correlation between low NK cell activity, *in vivo*, and high level human hepatocyte engraftment. We also showed that higher level engraftment of human hepatocytes was achieved in both NK cell-depleted SCID/Alb-uPA mice and Rag2$^{-/-}\gamma c^{-/-}$/Alb-uPA (T,B and NK cell deficient) mice compared with untreated SCID/Alb-uPA mice. These results support a critical role for mouse NK cells in the rejection of human hepatocytes xenotransplanted to immunodeficient mice.

Introduction

Xenotransplantation of human tissues/cells to receptive animal hosts facilitates investigation of human diseases and biology in an *in vivo* environment, with important advantages for insight into disease biology and into potential strategies for prevention and treatment. It has been reported that development of a humanized mouse model may provide an opportunity to study various human biological processes and liver-centric infections in human liver tissue while in an *in vivo* (murine) system [1].

Transgenic mice in which urokinase type plasminogen activator (uPA) transgene expression is targeted to hepatocytes by an albumin promoter (Alb-uPA) develop a form of subacute liver failure secondary to the toxic overexpression of uPA in hepatocytes [2,3]. Introduction of this transgene to a mouse with background immunodeficiency can be achieved by backcrossing with Swiss athymic nude mice [4], recombinase activation gene 2

(RAG-2) knockout mice [5], or SCID-beige mice [6]. Chimeric SCID/Alb-uPA mice have proven to be useful for the study of human hepatitis [6,7] and malaria [8,9], but may also be considered for other human hepatocyte-related medical and clinical studies as they harbor human liver cells in very large numbers and for prolonged periods. Such validation studies support the use of mice with chimeric human livers as an important model for exploration of new therapies and prevention strategies for liver based diseases [10–12]. Limitation of chimeric mouse models remain, however, including the fact that the level of human chimerism achieved in SCID/Alb-uPA mice after hepatocyte transplantation is highly variable [6,7,13] and that complete humanization has not been demonstrated.

It remains unclear if natural killer (NK) cell activity in the SCID/Alb-uPA mouse model has an important negative impact on the engraftment and expansion of human hepatocytes after transplantation, and as such, what the potency of NK cell activity is in the absence of T and B cell function.

Natural killer cells are a unique subset of lymphocytes that primarily contribute to antiviral and antitumor immune responses [14–16]. Natural killer cells have also shown a dominant role in the rejection of xenografts in human [17,18], nonhuman primate [19], and rodent systems [20,21]. Although recent studies, using organs from α1,3-galactosyltranseferase gene-knockout (GT-KO) homozygous pigs demonstrated some prolongation of survival of hearts [22], kidney [23], and lungs [24] in nonhuman primates, GT-KO pig offers no advantage in terms of NK cell-mediated rejection because the recognition of pig vascular endothelial cells by NK cells is not dependent on the expression of Galα1,3Gal (Gal) [25,26]. Therefore, understanding the role of NK cells in the rejection of xenografts may provide important insights and opportunities to assist in improvement of xenograft survival.

We report that NK cell activity is a primary limiting factor affecting human hepatocyte engraftment in T and B cell deficient mice and that significantly greater success is achieved in level of human chimerism within the mouse's liver with NK cell depletion in uPA mice, whether by treatment with exogenous agents (anti-NK cell antibody) or by genetic mechanisms (common gamma/Rag2 double knockout mice: $Rag2^{-/-}\gamma c^{-/-}$).

Materials and methods

Animals

BALB/c mice were purchased from the Frederick Cancer Research Facility (Frederick, MD, USA). B6 MHC class I-deficient (β2 microglobulin knockout) ($\beta2M^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Habor, ME, USA). Severe combined immunodeficiency and SCID-beige and common gamma (γc)/Rag2 double knockout mice ($Rag2^{-/-}\gamma c^{-/-}$) [27] were purchased from the Taconic Laboratory (German Town, NY, USA). Severe combined immunodeficiency/albumin linked-urokinase type plasminogen activator (uPA) (SCID/Alb-uPA) mice were generated from CB-17 mice and SCID-beige/uPA mice as described [6]. All animals were maintained in a specific pathogen-free micro-isolator environment, and were housed in micro-isolator cages containing autoclaved feed, bedding, and acidified water. Animal care was in accordance with the Canadian Council on Animal Care (1993) guidelines. Experimental protocols were reviewed and approved by the University of Alberta Health Sciences Animal Welfare Committee.

Generation of $Rag2^{-/-}\gamma c^{-/-}$/Alb-$uPA^{+/+}$ expressing transgenic mice

$Rag2^{-/-}\gamma c^{-/-}$ mice were crossed with SCID/Alb-uPA mice and backcrossed to generate $Rag2^{-/-}\gamma c^{-/-}$ homozygous for the Alb-uPA transgene ($Rag2^{-/-}\gamma c^{-/-}$/Alb-uPA). $Rag2^{-/-}\gamma c^{-/-}$ breeders were selected by PCR-based genotyping (as provided by Taconic) of genomic DNA extracted from tail tissue (DNeasy; Qiagen, Mississauga, ON, Canada). Zygosity of the Alb-uPA transgene was determined in offspring using a multiplex PCR procedure as described by Meuleman *et al.* [28].

Human hepatocyte isolation and transplantation

Ethical approval for human tissue was obtained from the University of Alberta, Faculty of Medicine Research Ethics Board, and informed consent was obtained from all human tissue donors. Hepatocytes were isolated and purified using collagenase-based perfusion, using previously described techniques [6]. Recipient mice (5–14 day-old) were anesthetized with isoflurane/O_2, and 1×10^6 viable hepatocytes were injected into the inferior pole of the spleen. Some SCID/Alb-uPA recipients received anti-asialo GM1 (AsGM1) (100 μl/mouse) (Cedarlane Laboratories, Hornby, ON, Canada) starting from day-1 for every 5 days until they were sacrificed.

Human α1 antitrypsin analysis

Samples of mouse serum (2 μl) were diluted 1/100 in blocking buffer and analyzed by ELISA using a polyclonal goat anti-human alpha-antitrypsin (hAAT) antibody (Diasorin, Stillwater, MN, USA) as described [7]. Level of

hAAT in mouse serum is a measure of the presence and activity of functional human hepatocytes (level of human chimerism).

In vivo ^{125}I-5iodo-2′-deoxyuridine (IUDR) assay

To measure NK cell activity *in vivo*, we used the ^{125}IUDR assay to measure early marrow proliferation in the spleen as recently described [29]. Briefly, a total of 5×10^5 bone marrow cells (BMC) from $\beta 2M^{-/-}$ mice were injected intravenously into lethally irradiated recipient mice that were or were not depleted of NK cells with a single injection of anti-AsGM1 1 day before bone marrow transplantation (BMT). Five days after BMT, the animals were injected i.p. with 25 μg 5-fluoro-2′-deoxyuridine (5-FU) and 3 μCi [^{125}I]-labeled iodo-2′-deoxyuridine (^{125}IUDR; ICN Biomedicals, Inc., CA, USA). Proliferation of donor BMC was measured by a γ-counter 20 h later. In principal, transplanted MHC class I knockout BMC home selectively to the spleen and serve as a specific target for NK cells.

In vitro ^{51}Cr release assay

Cytotoxic *in vitro* assays were performed as described [29]. Briefly, recipient NK cells were purified from the spleens of untreated SCID-beige, untreated $Rag2^{-/-}\gamma c^{-/-}$ mice and low hAAT SCID/Alb-uPA mice (low hAAT representing graft failure) after immunization with polyinosinic-polycytidylic acid (poly I:C) (Sigma, St. Louis, MO, USA) (150 μg/body) 1 day before assay. These splenic NK cells were co-cultured with ^{51}Cr-labeled Yac-1 cells at various effector:target (E:T) ratios. Culture supernatants were then harvested and ^{51}Cr releases determined with an automated γ-counter. Percent specific lysis (PSL) was calculated with the formula: PSL = [(experimental release−spontaneous release)/(maximum release−spontaneous release)] × 100%.

NK cell cytotoxicity assay against human hepatocytes

Freshly isolated hepatocytes from human patients who did not have liver cirrhosis were used as target cells. Spleen cells from control SCID-beige mice or from SCID/Alb-uPA mice that had rejected transplanted human hepatocytes by 6 weeks post-transplantation were used as effector cells with poly I:C (150 μg/body, i.p.) immunization 1 day before assay. After the human hepatocytes (5×10^3 cells/well) had been cultured with such spleen cells at an E:T ratio of 60, hepatocyte injury was evaluated by analyzing alanine aminotransferase (ALT) release. Alanine aminotransferase activity in each supernatant was assayed by standard enzymatic methods. Percent cytotoxicity was calculated as [(experimental ALT−spontaneous ALT)/(total ALT−spontaneous ALT)] × 100%.

Immunohistochemistry

Paraffin-fixed liver sections underwent antigen retrieval by proteinase K followed by goat serum blocking then staining with the anti-AsGM1, using the horseradish peroxidase procedure or the rat anti-mouse F4/80 monoclonal antibody (Abcam, Cambridge, MA, USA; A6640), using the avidin-biotin-peroxidase complex (ABC) procedure to identify mouse NK cells or mouse macrophage respectively.

Phenotypic assay for beige homozygosity

Our original uPA mouse colony was developed on a SCID-beige background to take advantage of the additional immunodeficiency from reduced NK cell activity associated with the beige trait. At present there is no genotypic test available for the bg^J allele used in the construction of our original SCID/Alb-uPA mouse strain [6]. This made tracking of the beige trait challenging as the colony expanded. We questioned whether the variability in hepatocyte engraftment we observed was due in part to variable presence of the beige trait in the mouse colony. A phenotypic assay for beige was performed by isolating fibroblasts from ear clip tissue as described by Shao *et al.* [30,31]. Nuclei were counterstained with 'Hoechst 33342' (Invitrogen, Carlsbad, CA, USA). Beige homozygotes are characterized by sparse but large, often pericentric lysosomes in contrast to the small lysosomes dispersed throughout the cytoplasm of wild type fibroblasts.

Statistics

The results were statistically analyzed by the Student *t*-test of means, the paired Student *t*-test or Mann–Whitney *U*-test when appropriate. A *P*-value <0.05 was considered to be statistically significant.

Results

Splenocytes recovered from SCID/Alb-uPA mice whose hepatocyte transplants have failed (been rejected) have NK cell activity, *in vitro*

To evaluate the NK cell activity of SCID/Alb-uPA mice that have demonstrated hepatocyte graft failure (defined as transplanted SCID/Alb-uPA mice having undetectable serum levels of hAAT at 6 weeks post-transplantation), we performed *in vitro* ^{51}Cr release cytotoxic assays against NK target Yac-1 cells. Similar NK cell-mediated killing

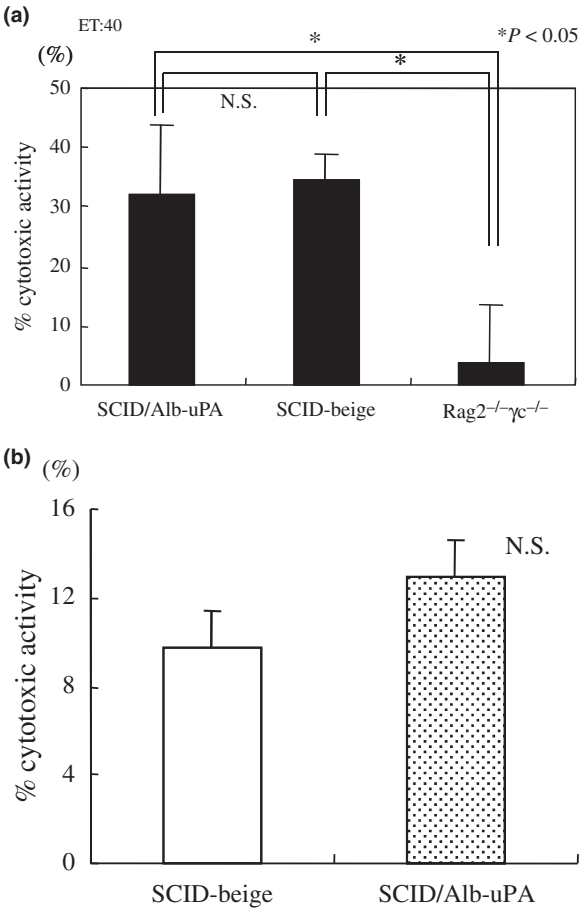

Figure 1 Severe combined immunodeficiency/albumin linked-urokinase type plasminogen activator (SCID/Alb-uPA) mice have natural killer cell activity *in vitro* cytotoxic assay. (a) Spleen cells were purified from SCID-beige (n = 5), Rag2$^{-/-}\gamma c^{-/-}$ (n = 5) and transplanted SCID/Alb-uPA animals that did not successfully engraft human hepatocytes (n = 5) after immunization with poly I:C (150 μg/body i.p.) 1 day before assay. These spleen cells were co-cultured with ^{51}Cr-labaled Yac-1 cell line at 40:1 effector:target (E:T) ratio in 96-well V-bottom plates for 4 h. (b) Hepatocytes were purified from human donors who underwent liver resection for colorectal metastasis. Liver tissues were not cirrhotic and patients had normal liver function. Spleen cells purified from SCID-beige (n = 5) and SCID/Alb-uPA animals that did not successfully engraft human hepatocytes (n = 5) were used as effector cells. Mice were immunized with poly I:C (150 μg/body i.p.) 1 day before assay. The results shown are the average ± SEM of values from triplicate samples. The results are representative of two similar experiments with different liver tissue donors.

activities were seen with failed SCID/Alb-uPA transplanted mice and with control untransplanted SCID-beige mice and both were significantly higher than with Rag2$^{-/-}\gamma c^{-/-}$ mice that lack T, B, and NK cells (Fig. 1a).

In vitro cytotoxic assays using normal human hepatocytes as targets also showed that SCID/Alb-uPA mice that had failed to successfully engraft human hepatocytes had significant and quantitatively similar cytotoxic activity towards co-cultured human hepatocytes as did SCID-beige mice (Fig. 1b).

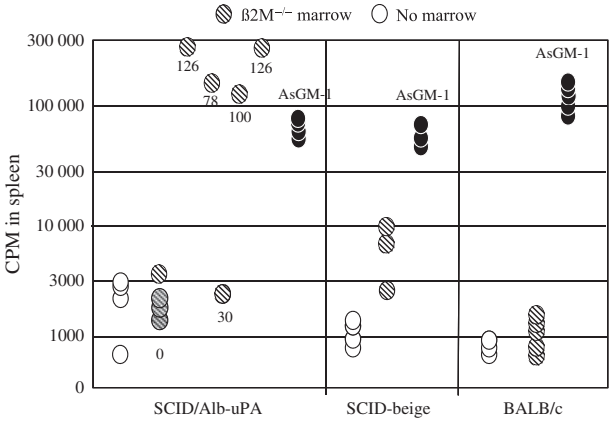

Figure 2 Low human alpha-1 antitrypsin (hAAT) severe combined immunodeficiency/albumin linked-urokinase type plasminogen activator (SCID/Alb-uPA) mice have normal natural killer (NK) cell function, while high hAAT SCID/Alb-uPA mice do not have NK cell function. No bone marrow cell (BMC) or β2M$^{-/-}$ BMC (5×10^5 cells/mouse) were transplanted into various groups of lethally irradiated recipient mice. SCID/Alb-uPA recipient mice underwent human hepatocyte transplantation 7–10 weeks before this assay. Weaning hAAT levels that were examined 6 weeks post-transplantation are shown below the symbols. The indicated groups received anti-AsGM1 treatment (100 μl/mouse, i.p). Each symbol represents an individual mouse.

NK activity is lower in high hAAT SCID/Alb-uPA human hepatocyte recipients than in low hAAT SCID/Alb-uPA recipients

To examine NK cell-mediated xenograft failure *in vivo*, ^{125}IUDR assays were performed in control SCID-beige mice and SCID/Alb-uPA recipient mice that received human hepatocyte transplantation 10 weeks prior. BALB/c mice with normal NK cell activity were also used as controls. In this assay, the rejection of transplanted MHC class I knockout marrow by NK cells is measured by the quantity of ^{125}IUDR-labeled BMC surviving in the spleen with and without prior NK cell depletion. In SCID-beige mice, NK cell depletion led to a significant increase in the level of ^{125}IUDR-labeled BMC associated with the spleen (Fig. 2). This data demonstrates that SCID-beige mice still have substantial *in vivo* NK cell activity, albeit, significantly lower than those of wild type BALB/c controls. In SCID/Alb-uPA human hepatocyte transplant recipients with human hepatocyte graft failure (hAAT = 0 μg/ml), MHC class I knockout marrow cells were rejected from spleens, at a level similar to untreated BALB/c mice. Prior NK cell depletion with anti-AsGM1 prevented this

rejection event. In contrast, MHC class I knockout marrow were retained in the spleens of high hAAT SCID/Alb-uPA, thereby demonstrating that, in contrast to failed (low hAAT) transplant recipients, high hAAT SCID/Alb-uPA recipients do not appear to possess significant numbers of active NK cells *in vivo*.

This evidence strongly supports a mechanism for the variable levels of human hepatocyte engraftment in SCID/Alb-uPA transplant recipients that is NK cell dependent.

Higher human hepatocyte engraftment was observed in Rag2$^{-/-}\gamma c^{-/-}$/Alb-uPA recipients than in SCID/Alb-uPA recipients

We generated Rag2$^{-/-}\gamma c^{-/-}$/Alb-uPA mice that have severe impairments in T-cell and B-cell development and function, and the complete absence of NK cell development. Hepatocytes were isolated from six human donors and transplanted into Rag2$^{-/-}\gamma c^{-/-}$/Alb-uPA ($n = 46$) mice and SCID/Alb-uPA mice ($n = 302$). Significantly higher serum hAAT levels were observed in Rag2$^{-/-}\gamma c^{-/-}$/Alb-uPA mice than in SCID/Alb-uPA mice (Rag2$^{-/-}\gamma c^{-/-}$/Alb-uPA mice versus untreated SCID/Alb-uPA: 56.3 ± 9.1 μg/ml vs. 37.5 ± 4.1 μg/ml, $P = 0.044$) at 6 weeks post-transplantation, further supporting the hypothesis that NK cells play a critical role in the rejection of transplanted human hepatocytes in the SCID/Alb-uPA mouse model (Fig. 3a). Moreover, the percentage of low hAAT mice (<10 μg/ml) representing profound graft failure was significantly lower in Rag2$^{-/-}\gamma c^{-/-}$/Alb-uPA mice than in untreated SCID/Alb-uPA recipients (Rag2$^{-/-}\gamma c^{-/-}$/Alb-uPA mice versus untreated SCID/Alb-uPA: 20.0 ± 3.4% vs. 61.1 ± 0.8%, $P = 0.0008$), indicating higher human hepatocyte engraftment in Rag2$^{-/-}\gamma c^{-/-}$/Alb-uPA mice (Fig. 3b), again indicating an important impact of NK cell function in determining the ultimate level of human hepatocyte engraftment and survival in uPA mice.

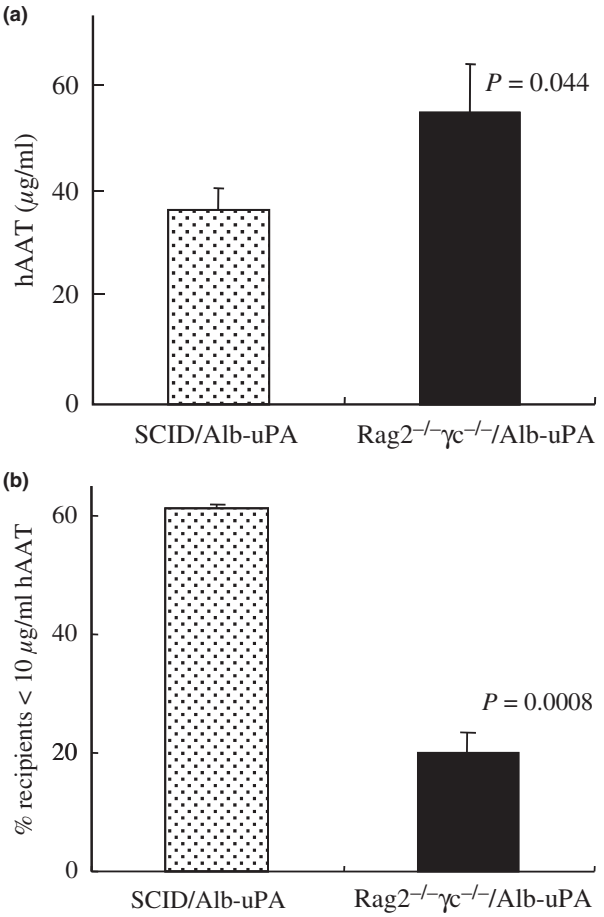

Figure 3 Higher engraftment of human hepatocytes was achieved in Rag2$^{-/-}\gamma c^{-/-}$/Alb-uPA mice than in severe combined immunodeficiency/albumin linked-urokinase type plasminogen activator (SCID/Alb-uPA) mice. (a) Human hepatocytes recovered from a series of six human liver tissue donors were transplanted into Rag2$^{-/-}\gamma c^{-/-}$/Alb-uPA ($n = 46$) mice or SCID/Alb-uPA mice ($n = 302$) in numbers determined for each experiment by the maximum numbers of potential recipient mice of each type in the colony. Serum levels of human alpha-1 antitrypsin (hAAT) were examined at 6 weeks post-transplantation by ELISA as show in materials and methods. The results shown are the average ± SEM of values from the data from seven different cohorts. (b) Percentage of low hAAT (<10 μg/ml) recipients, representing profound graft failure, is shown for the same mice as were examined in Fig. 3a. Seven different cohorts were examined in this assay. The percentage of low hAAT (<10 μg/ml) recipients was higher in SCID/Alb-uPA than in Rag2$^{-/-}\gamma c^{-/-}$/Alb-uPA mice at 6 weeks post-transplantation.

NK cell depleted-SCID/Alb-uPA recipient mice have higher engraftment of transplanted human hepatocytes than untreated SCID/Alb-uPA recipients

To directly address the role of NK cells in SCID/Alb-uPA mice, we compared NK cell-depleted SCID/Alb-uPA mice ($n = 109$) and untreated SCID/Alb-uPA ($n = 60$) mice that were recipients of hepatocytes isolated from three human donors. *In vivo* depletion of NK cells allowed higher engraftment of human hepatocytes in NK cell depleted-SCID/Alb-uPA mice. Serum hAAT in NK-depleted SCID/Alb-uPA mice was significantly higher than in untreated SCID/Alb-uPA mice at 6 weeks post-transplantation (NK cell-depleted SCID/Alb-uPA mice versus untreated SCID/Alb-uPA: 117.4 ± 13 μg/ml vs. 68.8 ± 17.6 μg/ml, $P = 0.028$) (Fig. 4a). By *in vivo* depletion of NK cells in SCID/Alb-uPA mice with anti-AsGM1, the percentage of low hAAT mice (<10 μg/ml) representing profound graft failure was significantly lower in

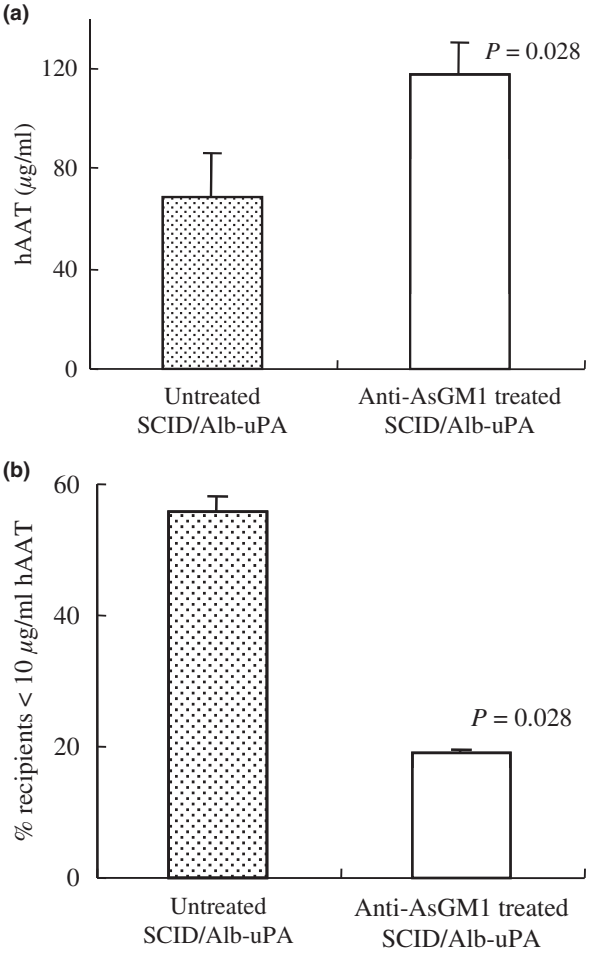

Figure 4 Natural killer (NK) cell depletion of recipients was effective in achieving higher engraftment of human hepatocytes in severe combined immunodeficiency/albumin linked-urokinase type plasminogen activator (SCID/Alb-uPA) mice. (a) Human hepatocytes recovered from a further series of three human liver tissue donors were transplanted into untreated SCID/Alb-uPA mice (n = 60) or NK cell-depleted SCID/Alb-uPA mice (n = 109). Anti-AsGM1 (20 μl/mouse, i.p.) was administrated from day-1 every 5 days until sacrifice for study. Serum levels of human alpha-1 antitrypsin (hAAT) were examined at 6 weeks post-transplantation by ELISA as show in materials and methods. The results shown in this assay are the average ± SEM of values from the data from three different cohorts. (b) The percentage of low hAAT (<10 μg/ml) recipients is shown. The same recipients were examined as in Fig. 4a. Three different cohorts were examined in this assay. The percentage of low hAAT mice (<10 μg/ml) representing graft failure was significantly lower in anti-AsGM1 treatment recipients than in untreated SCID/Alb-uPA recipients at 6 weeks post-transplantation.

anti-AsGM1 treated recipients than in untreated SCID/Alb-uPA recipients (NK cell-depleted SCID/Alb-uPA mice versus untreated SCID/Alb-uPA: 18.9 ± 0.59% vs. 55.9 ± 2.33%, P = 0.028) (Fig. 4b), indicating that NK cell depletion is effective in improving engraftment of transplanted human hepatocytes.

These results further demonstrate the importance of NK cells in the rejection of transplanted xenogeneic human hepatocytes independent of T or B cell support.

No homozygosity of beige mutation was detected in either SCID/Alb-uPA recipients with high engraftment of transplanted human hepatocytes or SCID/Alb-uPA recipients with low engraftment

Beige mice have been demonstrated to have reduced NK cell activity, although NK cells are normally present. It is theorized that the large granules containing cytotoxic agents including perforin and granzymes fail to be secreted as part of NK cell immune attack. To examine the possibility of homozygosity of beige mutation presence or absence explaining the difference between successful (high hAAT) and unsuccessful (low hAAT) transplanted SCID/Alb-uPA recipient mice, we performed a phenotypic assay for beige homozygosity. We used SCID-beige mice and SCID mice as positive and negative controls respectively. As shown in Fig. 5, the characteristic giant lysosomes, characteristic of mice that are homozygous for the beige mutation, were present in SCID-beige mice, but were absent in both high hAAT and low hAAT SCID/Alb-uPA recipients and SCID negative control mice. These results indicate that all SCID/Alb-uPA recipients from our colony utilized in these experiments are phenotypically distinct from the original homozygous SCID-beige mice from which they were derived. It appears likely that this altered phenotype contributes to increased NK cell activity in our animal colony.

Discussion

Hepatocyte transplantation is a potential solution to overcome chronic shortages of donor organs, as the hepatocytes have a large regenerative capacity and therefore in theory, a single liver donor could provide hepatocytes for multiple recipients [32]. The long-term clinical promise of hepatocyte transplantation is complemented by the expanding use of chimeric animal models as tools for study of human disease and of human biology and metabolism [7,11,12,33]. However, improving and stabilizing engraftment and repopulation of transplanted human hepatocytes in existing transgenic mouse models remains a significant obstacle. A recent study showed that complement inhibitor and macrophage depletion of fumarylacetoacetate hydrolase (Fah)-deficient and Rag2/common γ-chain of the interleukin2 receptor knockout ($Fah^{-/-}/Rag2^{-/-}/Il2\gamma g^{-/-}$) mice was effective in achieving high level hepatocyte repopulation without

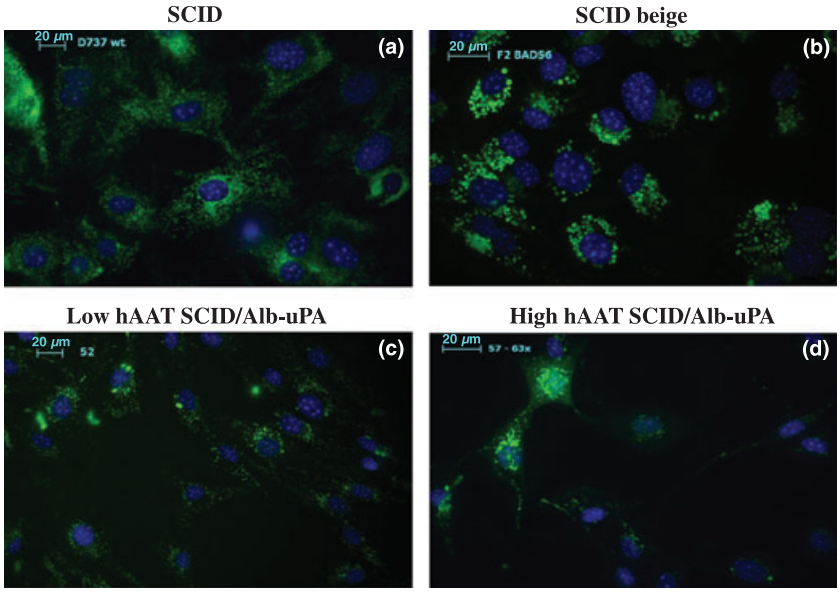

Figure 5 Neither severe combined immunodeficiency/albumin linked-urokinase type plasminogen activator (SCID/Alb-uPA) recipients with higher engraftment of human hepatocytes nor those with lower engraftment are homozygous for beige. Fibroblasts from ear clips of mice of the indicated genotype were isolated, their lysosomes stained with Lucifer Yellow-CH and their nuclei with Hoechst 33342, and examined by fluorescent microscopy. (a) SCID: note the many small, diffusely distributed lysosomes. (b) SCID-beige: note the large lysosomes characteristic of beige homozygotes. (c) SCID/Alb-uPA recipients with lower engraftment of human hepatocytes. (d) SCID/Alb-uPA recipients with higher engraftment of human hepatocytes. In both panels c and d, the lysosomes resemble the wild type rather than the beige phenotype.

uPA-expression in recipients [34]. Fah$^{-/-}$/Rag2$^{-/-}$/Il2γg$^{-/-}$ mice are lacking T, B, and NK cells.

Our current studies focused on the role of NK cells in humanized transgenic mouse models. Natural killer cells appear to play a critical role in the rejection of xenografts, lacking species specific MHC class I which interact with killer inhibitory receptors of NK cells [35]. Natural killer cells may contribute to xenograft rejection through both direct and indirect mechanisms. For example, human NK cells lyse target porcine endothelial cells with activation via NK activating receptor such as NKG2D [36]. Human NK cells also activate porcine endothelial cells by induction of adhesion molecules, promoting invasion of immune cells and enhanced cytokine secretion such as IFN-γ [37]. In xenogeneic hepatocyte transplantation, we focused on direct NK cell rejection mechanisms, because a hepatocyte graft is initially a nonvascularized xenograft.

As SCID/Alb-uPA mice have NK cell activity as shown in Fig. 1a, we compared the human hepatocyte engraftment in SCID/Alb-uPA mice depleted of NK cells by anti-AsGM1 treatment with untreated SCID/Alb-uPA mice. The proportion of unsuccessful engraftments of transplanted human hepatocytes was much higher in untreated SCID/Alb-uPA mice and higher serum hAAT levels were achieved in NK cell-depleted SCID/Alb-uPA mice (Fig. 4a and b), indicating higher levels of human hepatocyte engraftment were observed in NK cell-depleted SCID/Alb-uPA mice. Other investigators have reported improvement of engraftment levels of human hepatocytes following depletion of both macrophage and NK cells in SCID/Alb-uPA recipients which already lack T and B cells [9]. As such, macrophages also appear capable of playing an important and quite independent role in the rejection of xenotransplanted human hepatocytes. Furthermore, we also showed that higher levels of repopulation and engraftment with transplanted human hepatocytes was evident at 6 weeks post-transplant in Rag2$^{-/-}\gamma$c$^{-/-}$/Alb-uPA mice than in non-NK cell depleted SCID/Alb-uPA mice (Fig. 3a and b). These results are consistent with a report that high levels of T-cell chimerism were observed after intravenous transfer of human peripheral blood mononuclear cells (huPBMCs) in Rag2$^{-/-}\gamma$c$^{-/-}$ mice, while intravenous transfer of up to 10^8 huPBMCs into SCID mice (that have NK cells) has been consistently ineffective [38].

We next investigated the level of NK cell activity of control SCID-beige mice and of SCID/Alb-uPA mice that had either good human hepatocyte engraftment or no human hepatocyte engraftment. Beige mice display defective natural killing [39]. However, the effect of the beige mutation on NK cells is neither selective nor complete: it affects the granules of many non-NK cell types and it does not ablate other NK cell activities such as

cytokine production [40,41]. Our *in vivo* ^{125}IUDR assay demonstrated that SCID-beige mice still possess moderate NK cell activity thereby facilitating the rejection of MHC class I knockout marrow, although, as expected, the activity was much lower than in wild-type BALB/c mice (Fig. 2). The results of this study indicate that human hepatocytes or other xenogeneic cell types transplanted into SCID-beige mice may be rejected via a T and B cell independent NK cell-mediated cytotoxic mechanism: although NK cell function is markedly reduced in mice with the 'beige' mutation, a low level of NK cell killing is present in SCID-beige mice. Although we performed immunohistochemistry to identify recipient mouse NK cells in the livers of high hAAT and low hAAT SCID/Alb-uPA recipients, we did not see significant differences in terms of NK cell numbers between them (data not shown). This followed our expectations because of the normal ability of NK cell numerical accumulation in SCID-beige mice, but impaired ability of NK cell cytotoxicity. Hemizygosity of the beige mutation in these mice may provide an important impact on NK cell function, but neither genotypic nor phenotypic assay is able to reliably differentiate the hemizygous state for beige.

Natural killer cells can kill hematopoietic tumors and virally infected cells by such key mechanisms as perforin/granzymes, tumor necrosis factor-family members (Fas ligand, TNF-related apoptosis-inducing ligand; TRAIL), nitric oxide signaling or IFN-γ [15]. Rejection of MHC class I knockout marrow in SCID-beige mice as demonstrated in the ^{125}IUDR assay suggests that these cells were rejected by perforin/granzyme-independent mechanisms, because the 'beige' mutation results in reduction of perforin/granzyme-dependent cytotoxic mechanisms [42]. This result is consistent with a previous report, showing that xenogeneic pig kidney cell lines (PK15) were rejected in anti-CD4/CD8 mAb-treated perforin knockout mice that have no T cells and no perforin-mediated NK cell cytotoxic activity, but have other cytotoxic mechanisms of NK cells [43]. It has been reported that human islet cells are accepted in nude mice that have no T-cell activity, but have NK cell activity [44]. In the current study, human hepatocytes were rejected by mouse NK cells *in vivo* and *in vitro*.

We also showed both high and low hAAT SCID/Alb-uPA recipients evaluated had no evidence of homozygosity of recessive beige mutation, as none of the animals have the enlarged perinuclear granules that are characteristic of bg/bg (Fig. 5). The animals in these studies were either hetero or null (wild type) for the beige mutation. This appears to have been a spontaneous development after many generations of breeding where a selective survival advantage is presumably associated with the loss of the additional immunodeficiency associated with beige, given the mice in the colony were originally generated from homozygous SCID-beige founders. As a result of lack of a reliable and efficient molecular assay for beige, maintenance of homozygous beige status in our colony was challenging .

Interesting results were obtained from the *in vivo* ^{125}IUDR assay, showing that high hAAT SCID/Alb-uPA mice have low NK cell activity and that low hAAT SCID/Alb-uPA mice have high NK cell activity (Fig. 2). These results demonstrate that an NK cell-mediated cytotoxic mechanism in SCID/Alb-uPA mice plays a critical role in the rejection of human hepatocytes. It has been reported that NK activity *in vivo* and *in vitro* is less in homo (beige,beige) < hetero (beige,+) < wild type (+,+) [45,46]. Although there is no reliable assay for hetero beige status versus no beige mutation, these results would provide support for the possibility that high hAAT animals from our colony are hetero for the beige mutation as they accept MHC class I knockout marrow and human hepatocytes better than low hAAT animals.

Overall, NK cells appear to remain an important barrier for overcoming rejection in xenotransplantation. Some investigators have succeeded in blocking NK cell inhibition against porcine endothelial cells by expression of HLA class I molecules, such as Cw3, E or G, but this protection appears only partial [47–49]. It might be necessary to identify the activating receptors and their corresponding ligands such as NKp44 and NKG2D which are critical for NK cell activation in pig to human xenogeneic combination [50]. Recently, we have demonstrated that xenogeneic BMT induced NK cell hyporesponsiveness in rat to mouse *in vivo* model [29]. Such advances may support prolonged xenograft acceptance and survival.

In conclusion, we have demonstrated that recipient mouse NK cells can play a critical role in the rejection of xenotransplanted human hepatocytes in transgenic mouse models. Further studies are necessary to confirm the exact pathway involved in NK cell dependent rejection mechanisms of human hepatocytes. Clarification of the role of NK cells in human:mouse chimeras may yield important insights for future studies in cell xenotransplantation, experimental or clinical. Furthermore, such understanding should facilitate development of improved/higher yield transgenic mouse models where human hepatocyte transplants will engraft and more effectively and reliably repopulate the murine liver to higher levels. This has important implications for enhanced utility of chimeric mouse models for such diverse applications as evaluation of drug metabolism by or toxicity to human liver *in vivo*, or for evaluation of the impact of new drug therapies on important liver centered disease processes such as

hepatitis C and malaria using human tissue in an *in vivo* test-bed.

Authorship

N.M.K.: participated in research design, data analysis, writing of the paper. T.K.: participated in research design, performed research, data analysis, and writing of the paper. D.D.: participated in research design. J.L.: participated in the performance of the research. G.L.: participated in the performance of the research. W.A.: participated in the performance of the research. T.C.: participated in the data analysis. D.L.T.: participated in the data analysis.

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