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Expression of human cytokines dramatically improves reconstitution of specific human-blood lineage cells in humanized mice

Qingfeng Chen², Maroun Khoury¹,², and Jianzhu Chen¹,²,1

¹Interdisciplinary Research Group in Infectious Diseases, Singapore-Massachusetts Institute of Technology Alliance in Research and Technology, S16-05-08, 3 Science Drive 2, Singapore 117563; and ²The Koch Institute for Integrative Cancer Research and Department of Biology, Massachusetts Institute of Technology, E17-131, 77 Massachusetts Avenue, Cambridge, MA 02142

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Adoptive transfer of human hematopoietic stem cells (HSCs) into mice lacking T, B and natural killer (NK) cells leads to development of human-blood lineage cells in the recipient mice (humanized mice). Although human B cell reconstitution is robust and T cell reconstitution is reasonable in the recipient mice, reconstitution of NK cells and myeloid cells is generally poor or undetectable. Here, we show that the poor reconstitution is mainly the result of a deficiency of appropriate human cytokines that are necessary for the development and maintenance of these cell lineages. When plasmid DNA encoding human IL-15 and Flt-3/Flik-2 ligand were delivered into humanized mice by hydrodynamic tail-vein injection, the expression of the human cytokine lasted for 2 to 3 weeks and elevated levels of NK cells were induced for more than a month. The cytokine-induced NK cells expressed both activation and inhibitory receptors, killed target cells in vitro, and responded robustly to a virus infection in vivo. Similarly, expression of human GM-CSF and IL-4, macrophage colony stimulating factor, or erythropoietin and IL-3 resulted in significantly enhanced reconstitution of dendritic cells, monocytes/macrophages, or erythrocytes, respectively. Thus, human cytokine gene expression by hydrodynamic delivery is a simple and efficient method to improve reconstitution of specific human-blood cell lineages in humanized mice, providing an important tool for studying human immune responses and disease progression in a small animal model. Humanized mouse | natural killer cells | myeloid cells | cytokine genes | hydrodynamic gene delivery

There has been a great need to study human immune responses to pathogen infections in a small animal model in a systematic and controlled manner. Over the past two decades, tremendous efforts have been devoted to reconstitute severe combined immunodeficient (scid) mice, which lack T and B lymphocytes, with human-blood lineage cells (1). However, early attempts were unsuccessful because of poor engraftment, rapid disappearance of human T and B cells, or rapid development of hematopoietic malignancies in recipient mice. A breakthrough was achieved by using recipient mice that are deficient not only in T and B cells, because of either scid mutation or mutation of the recombination activating gene (Rag), but also in natural killer (NK) cells because of deletion of the common gamma chain (γc or Il2rg) (2, 3). Adoptive transfer of human hematopoietic stem cells (HSCs) into either NOD-scid Il2rg−/− (NSG) recipients or BALB/c-Rag2−/− Il2rg−/− recipients leads to stable, long-term engraftment of HSCs in the recipient bone marrow (BM) and generation of all human-blood lineage cells in the periphery (humanized mice or humice) (2, 3).

The existing humanized mouse models provide an important tool to study infection by human pathogens (4–9), especially those that infect human-blood lineage cells. They also begin to allow investigations of human immune responses to pathogens in a small animal model. However, the current models are far from optimal. For example, the level of human cell reconstitution differs markedly among different cell lineages. The reconstitution of B cells is robust, the reconstitution of T cells is reasonable, but the reconstitution of NK cells and myeloid lineage cells is generally poor or undetectable. Recently, significant reconstitution of human dendritic cells (DC) and monocytes/macrophages was reported in NOD-scid mice that were engrafted with human fetal thymus, liver, and autologous human CD34+ cells (BLT mice) (10). Still, human NK cells were absent in BLT mice. As NK cells and myeloid cells play important roles in innate immune responses, development of humanized mice with adequate levels of reconstitution of these cell types is critical for realizing the full potential of the humanized mouse models in infectious disease research and other research involving blood lineage cells.

All blood cell lineages are derived from common HSCs. Cytokines play a key role during their differentiation and maintenance. For example, IL-15 is required for development and survival of NK cells (11), GM-CSF and IL-4 for DC development (12), macrophage colony stimulating factor (M-CSF) for monocyte/macrophage development and maintenance (13), and erythropoietin (EPO) and IL-3 for erythrocyte development (14). However, because of evolutionary divergence between human and mouse, these cytokines are species-specific (i.e., the mouse cytokines do not function on human cells). For example, mouse IL-15 has no effect on human NK cells and precursors (15), resulting in poor reconstitution of human NK cells in humice (16, 17). Similarly, mouse GM-CSF, IL-4 (18, 19), M-CSF (20), and IL-3 (21) have all been reported not to function on human cells. Thus, it is likely that the poor reconstitution and function of NK cells and myeloid cells in humice are a result of the lack of specific human cytokines. Expression of human cytokines in the reconstituted mice should stimulate differentiation, survival, and function of specific human-blood lineage cells.

We have tested this hypothesis and developed a simple and efficient method to improve the reconstitution of specific human-blood lineage cells in humanized mice. By hydrodynamic delivery of DNA vectors encoding human IL-15 and Flt-3/Flik-2 ligand (FL), we show that specific human cytokines can be detected in the circulation of humice for 2 to 3 weeks. As a result, a significantly elevated number of human NK cells is observed in various organs for more than a month. The cytokine-induced NK cells are fully functional both in vitro and in vivo. Using the same strategy, the reconstitution level of human DCs, monocytes/macrophages, and erythrocytes can also be greatly enhanced in humice. Our study demonstrates that the poor reconstitution of NK cells and myeloid...
cells in the current model of humanized mice is the result of a lack of appropriate human cytokines required for their differentiation and maintenance, and that hydrodynamic delivery of human cytokine genes is a simple and efficient method to overcome the poor reconstitution of these cell lineages.

Results

Stimulation of NK Cell Differentiation by Human Cytokines in Vitro. To construct humanized mice, CD34+ HSCs isolated from human cord blood were adoptively transferred into sublethally irradiated NSG pups. Twelve weeks after reconstitution, mononuclear cells (MNCs) from peripheral blood were stained with antibodies specific for human CD45 and mouse CD45 (Fig. S1). The average reconstitution rate was ~50% in the blood [reconstitution rate = %CD45+ human cell/(%CD45+ human cell + %CD45+ mouse cell)]. Among the CD45+ human leukocytes, the level of CD19+ B cells ranged from 40 to 85% and the level of CD3+ T cell ranged from 10 to 50%. Although NK cells were detected in the blood, BM, spleen, lung, and liver, their frequency was significantly lower than that in the corresponding human tissues or mouse tissues (see Fig. S1).

To determine the cause underlying the poor NK cell reconstitution in humice, we tested whether human CD34+ cells from the BM of humice can be stimulated by human IL-15 and FL to differentiate into NK cells in vitro. FL stimulates differentiation of multiple hematopoietic cell lineages, including CD34+ NK progenitors that can respond to IL-15 (22). The combination of FL and IL-15 is expected to favor the differentiation of CD34+ precursors toward NK cells. Thus, purified human CD34+ cells (>80%) (Fig. 1A) from humice BMs were cultured for 7 days in the presence of FL and IL-15 and analyzed for expression of NK cell markers CD56 and NKP46. In the presence of the cytokines, ~11% of cells were positive for both CD56 and NKP46, whereas few very cells were positive in the absence of the cytokines (Fig. 1B). These results suggest that CD34+ human cells in the BM of humice are capable of differentiating into NK cells if the appropriate cytokine environment is provided.

Expression of Human Cytokines in Mice by Hydrodynamic Injection of Plasmid DNA. The finding that human NK cells developed in vitro in the presence of IL-15 and FL suggests that these human cytokines might also stimulate NK cell development in humice. One way to introduce human cytokines into mice is by daily injection of recombinant proteins. Because this way is cumbersome and expensive, we expressed human cytokines in mice by hydrodynamic delivery of cytokine-encoding DNA plasmid. Human IL-15 has an unusually long signal peptide sequence (45 aa residues), which is known to lead to poor secretion of IL-15 (23). To increase the level of IL-15 secretion, we constructed an IL-15-expressing vector in which the IL-15 signal peptide was replaced by the signal peptide of human IL-2 (Fig. S2A). This replacement increased the serum level of IL-15 ~100-fold (Fig. S2B). With a single hydrodynamic injection of 50 μg IL-15-encoding plasmid, a high level of IL-15 was detected in the serum 1 day after injection and a significant level was maintained for 14 days (Fig. 2). Similarly, a single injection of FL-encoding plasmid resulted in expression of FL in the serum for 21 days. Thus, hydrodynamic delivery of cytokine genes is a simple and efficient method to introduce human cytokines in mice.

Enhanced Reconstitution of Human NK Cells Following IL-15 and FL Gene Delivery. To determine the effect of IL-15 and FL expression on NK cell development, 9 days after gene delivery humice were analyzed for NK cell reconstitution in various organs by flow cytometry. Injection of empty pcDNA vector or FL-encoding vector did not significantly affect the frequency of CD56+ NK cells (Fig. 3A). However, expression of IL-15 significantly increased the frequency of CD56+ NK cells in the blood, spleen, BM, lung, and liver (Fig. 3 A and B). The increase in frequency of NK cells was even more dramatic when both IL-15 and FL were expressed in humice, reaching the level observed in normal human peripheral blood (5%–21% of leukocytes) (24) and normal mouse tissues (25). Corresponding to the increased frequency of NK cells, the absolute numbers of NK cells were markedly increased in the spleen and BM (Fig. S3B). Furthermore, the elevated frequency of CD56+ NK cells in the blood was maintained for at least 30 days after gene delivery (Fig. 3C). In addition, cytokine-induced NK cells expressed many of cell surface receptors known to be important for NK cell function (Fig. S4), including the activating receptor NKG2D, inhibitory receptors NKG2A, CD94, and KIR, the natural cytotoxicity triggering receptor NKP46, the NK cell marker CD7, the early activation marker CD69, and the Fc receptor CD16. These results indicate that cytokine-induced NK cells exhibit the characteristic surface phenotype of normal NK cells.

In addition to stimulating NK cell development, both FL and IL-15 are known to exert effect on other hematopoietic cell lineages (26–29). Thus, cells from spleen, BM, lung, and liver of humice were enumerated and analyzed by flow cytometry. Expression of IL-15 and FL also induced significant increase in CD14+ monocytes/macrophages, CD11c+CD16+ myeloid dendritic cells, ILT7+CD303+ plasmacytoid dendritic cells, and CD19+ B cells in the spleen and BM (see Fig. S3). These results demonstrate that expression of human IL-15 and FL dramatically improves the reconstitution of NK cells as well as other myeloid and lymphoid cells in humanized mice.

Cytokine-Induced NK Cells Are Functional. We investigated whether cytokine-induced human NK cells are functional (i.e., able to kill...
target cells and express IFN-γ following appropriate stimulation). CD56+ NK cells were purified from the BM and spleen of IL-15- and FL-treated mice. When mixed with MHC class I-deficient target cells K562, we observed an increased level of target cell lysis with increasing numbers of NK cells added (Fig. S5A). When purified NK cells were stimulated with a Toll-like receptor 3 agonist poly(I:C), which is known to activate NK cells to produce proinflammatory cytokines (30), IFN-γ was detected in the culture supernatant (Fig. S5B). In the presence of human DCs, the level of IFN-γ secretion was further increased. When poly(I:C) was injected into humanized mice, a significantly increased level of IFN-γ was detected in the serum of humice that were injected with cytokine-encoding DNA compared to the noninjected humice (Fig. S5C).

We also challenged humice with adenovirus, which is known to cause NK cell-dependent liver damage (31). Nine days after cytokine gene delivery, replication-deficient adenovirus was intravenously injected into humice. Three days later, the liver was harvested and stained with H&E. Abundant leukocyte infiltration and large areas of necrosis were observed in the livers of IL-15- and FL-treated adenovirus-infected humice. However, nontreated humice infected with adenovirus exhibited only mild cell infiltration and damage (Fig. 4A). Correspondingly, the serum alanine aminotransferase (ALT) level was significantly elevated in IL-15- and FL-treated adenovirus-infected humice (Fig. 4B). This increase was correlated with an approximately fourfold increase in serum IFN-γ level (Fig. 4C) and an approximately fivefold increase in infiltrating human leukocytes in the livers (Fig. 4D). Immunohistochemical analysis of liver slices confirmed localization of CD56+ NK cells within the lesions (Fig. 4E). These results strongly suggest that cytokine-induced human NK cells are functional.

Improving Reconstitution of Other Human-Blood Cell Lineages. We tested whether cytokine gene delivery can be used as a general method to improve reconstitution of specific human-blood cell lineages in humice. In culture, human CD34+ cells purified from the BM of humice were stimulated to differentiate into CD11c+CD209+ DCs by GM-CSF and IL-4, into CD14+ monocytes/macrophages by M-CSF, and into CD235a+ erythrocytes by EPO and IL-3 (Fig. S6). In vivo, hydrodynamic delivery of DNA vectors expressing GM-CSF, IL-4, and FL into humice resulted in markedly increased frequency of CD11c+CD209+ DCs in the blood, spleen, BM, lung, and liver (Fig. S4). Similarly, expression of M-CSF led to improved reconstitution of CD14+ monocytes/macrophages in both lymphoid and nonlymphoid organs (Fig. 5B). Expression of EPO and IL-3 resulted in the appearance of CD235a+ human erythrocytes in the blood (Fig. 5C), reaching 3 to 5% of all red blood cells. Thus, cytokine gene expression by hydrodynamic injection of DNA plasmids is a general and efficient method to improve reconstitution of specific human-blood cell lineages in humice.

Discussion

Development of humanized mice provides an opportunity to overcome ethical and many technical constraints on the in vivo study of human cells and tissues. However, reconstitution of NK cells and myeloid cells are generally poor in the humanized mouse models using NSG or BALB/c-Rag2−/− Il2rg−/− mice as recipients. In BLT mice, human NK cells and RBC are absent, despite significant reconstitution of DCs and monocyte/macrophage. We noticed that many cytokines, including IL-15, GM-CSF, IL-4, M-CSF, and IL-3, required for NK cell or various myeloid cell development and maintenance, show significant sequence divergence between human and mouse. Previous studies have documented that these murine cytokines have little effect on appropriate human cell types. Because these cytokines are predominantly produced by nonhematopoietic cells, the lack of these human cytokines could explain the poor reconstitution of NK cells and myeloid cells in humice.

Supporting this interpretation, we showed that human CD34+ precursor cells isolated from the BM of humice can be stimulated in vitro to differentiate into NK cells, DCs, monocytes/macrophages, and erythrocytes. When appropriate human cytokines are introduced in the humanized mice by hydrodynamic delivery of cytokine-encoding plasmid DNA, significantly elevated levels of NK cells, DCs, monocytes/macrophages, and erythrocytes are induced. As the serum level of cytokines declines, the level of reconstitution also declines. Thus, the poor reconstitution of NK cells and myeloid cells in humice is a result of the lack of appropriate human cytokines normally required for their differentiation and maintenance. Introduction of appropriate cytokines leads to a dramatic increase in reconstitution levels of these human-blood cell lineages in humice.

Hydrodynamic gene delivery is widely used to produce high level, transient hepatic and systemic transgene expression in mice (32). The method involves tail-vein injection of DNA in a large volume (10% body weight) in a short duration (6–8 s). The hydrodynamic pressure causes liver damage, leading to uptake of DNA by hepatocytes (32). Following transcription and translation, cytokines are secreted into the circulation and can reach the target cells in the BM or other organs. Thus, with a single injection of cytokine encoding DNA, IL-15 was detected in the serum for 2 weeks and FL for 3 weeks. The
difference between serum duration of IL-15 and FL is probably because of difference in the protein’s half-life or that IL-15 is normally bound on the cell surface via IL-15Rα chain (33). The amount of IL-15 and FL produced from a single DNA injection is apparently sufficient to induce a markedly elevated level of NK cells for at least 30 days. The persistence of NK cells when the cytokines were no longer detected in the circulation indicates that the critical role of the cytokines is exerted at an early stage of the differentiation. Once generated, NK cells are able to survive for an extended time after cytokines become undetectable in the circulation. Because of their effect on multiple blood-cell lineages, expression of FL and IL-15 also lead to elevated levels of monocytes/macrophages, DCs, and B cells, but not T cells, in the spleen and BM. Furthermore, expression of appropriate cytokines by hydrodynamic gene delivery also markedly enhances the reconstitution of specific myeloid lineage cells, including DCs, monocytes/macrophages, and erythrocytes, demonstrating the broad utility of the approach. Compared to the improved reconstitution of NK cells, DCs and monocytes/macrophages, which became apparent 7 days after delivery of human cytokine genes, improved reconstitution of erythrocytes did not reach the peak level until 30 days after cytokine gene delivery. This can be explained by the difference in the ratios of human WBC versus mouse WBC on the one hand, and human RBC versus mouse RBC on the other. Because of the large numbers of mouse RBC, it requires a longer time to produce sufficient numbers of human RBC to reach a similar percentage. Previously, two groups have reported enhanced NK cell development by injecting recombinant human IL-15 into NOD-scid mice or BALB/c-Rag2−/−Il2rg−/− mice (16, 17). Compared to the daily cytokine injection, which is cumbersome and expensive, expression of cytokine genes by hydrodynamic gene delivery is an affordable, simple, and efficient method, as a single injection leads to elevated reconstitution of specific blood-cell lineages for more than 30 days.

The cytokine-induced NK cells exhibit normal surface phenotype and function. In contrast to a previous observation, where human NK cells were generated following daily injection of recombinant IL-15 in NOD-scid mice, the cells expressed NKp46 but not NKG2D and NKG2A (17). In the present study, cytokine-induced human NK cells expressed all three major families of NK receptors, including activating receptor NKG2D, inhibitory receptors NKG2A and KIR, and the natural cytotoxicity receptor NKp46. Consistently, cytokine-induced NK cells are capable of lysing MHC class I-deficient target cells and secreting IFN-γ upon poly(I:C) stimulation both in vitro and in vivo. Furthermore, cytokine-induced NK cells are capable of mounting a robust response against adenovirus infection as indicated by the extensive liver necrosis and the high level of serum ALT in IL-15- and FL-treated humice. Similar to wild-type NK cells in mice, which mediate hepatitis by IFN-γ secretion (31, 34), the levels of serum IFN-γ of IL-15- and FL-treated adenovirus-infected humice were significantly elevated. These findings suggest that cytokine-induced NK cells are normal in both surface phenotype and function.

Many infectious diseases in humans are caused by pathogens that infect specific human-blood lineage cells. For example, dengue virus infects DCs and monocytes/macrophages (35, 36); *Plasmodium falciparum* infects RBC; Epstein Barr Virus infects B cells, and human immunodeficient virus-1 infects macrophages and T cells. The establishment of a robust human model for these infections in mice is hindered by either the absence of the pathogen target cells, such as RBC, or by their presence in much lower numbers than in human tissues. The ability to enhance reconstitution of specific human-blood cell lineages in humanized...
mice by hydrodynamic delivery of cytokine genes will help to make the humanized mice a better model for these human pathogen infections and immune responses against the pathogens. As NK cells and myeloid cells are also involved in other diseases, improved reconstitution of these cell types will facilitate the study in mice of these disease processes.

**Materials and Methods**

**HSC Isolation, Construction of Humanized Mice, and Hydrodynamic Gene Delivery.** Human cord blood was obtained from Singapore Cord Blood Bank. Cord blood MNCs were separated by Ficoll-Hypaque density gradient. CD34⁺ cells were purified with the RosetteSep system according to the manufacturer’s protocol (Stem Cell Technologies). The purity of CD34⁺ cells was >95%. To expand HSCs, purified CD34⁺ cells were cultured for 11 to 14 days in serum-free medium in the presence of defined factors (37). Both unexpanded and expanded HSCs were used to generate humanized mice.

NSG mice were purchased from the Jackson Laboratories and maintained under specific pathogen-free conditions in the animal facilities at Nanyang Technological University and National University of Singapore. To reconstitute mice, newborn pups (less than 48 h old) were irradiated with 100 cGy using a Gamma radiation source and injected intracardially with CD34⁺CD133⁺ cells (1 × 10⁶ cells/recipient). Human cytokine genes were cloned separately into pcDNA3.1⁺ vector (Invitrogen). Plasmid DNA was purified by Maxi-prep Kit (Qiagen). For hydrodynamic gene delivery, 12-week-old humice were injected with 50 μg of each plasmid in a total of 1.8-ml saline within 7 s using a 27-gauge needle. All research with human samples and mice was performed in compliance with the institutional guidelines of the National University of Singapore and Nanyang Technological University.

**Single Cell Preparation, Antibodies, and Flow Cytometry.** Single-cell suspensions were prepared from spleen and BM by standard procedures. To isolate MNCs from humice liver, the liver was pressed through a 200-gauge stainless steel mesh and debris was removed by centrifugation at 50 × g for 5 min. Supernatants containing MNCs were collected, washed in PBS, and resuspended in 40% Percoll (Sigma) in RPMI medium 1640. The cell suspension was gently overlaid onto 70% Percoll and centrifuged at 750 × g for 20 min. MNCs were collected from the interphase, washed twice in PBS, and resuspended in 40% Percoll in RPMI medium 1640. The cell suspension was passed through a 200-gauge stainless steel mesh, and MNCs were isolated with Percoll centrifugation as described above.

The following antibodies were used: CD3 (SK7), CD34 (5B1), CD19 (HIB19), NKGD2 (1D11), NKp46 (9E2), CD94 (DX23), CD16 (5G8), CD56 (B159), HLA-DR (L243), GM-CSF (ME02), CD11c (B9), CD209 (DC-SIGN), CD303 (AC144), CD14 (M-T01), CD45 (2D1), CD69 (7B8), CD33(WM53) from Becton-Dickson; KIR2DL2L3 (DX27), ILT7 (17G10.2) and CD235ab (H20841) from Miltenyi Biotec; CD159a (NKGA2A), Z199 from Beckman Coulter; and CD133 (EMK08) and mouse CD45.1 (A20) from ebioscience. Cells were stained with appropriate antibodies in 100-μl PBS containing 0.2% BSA and 0.05% sodium azide for 30 min on ice. Flow cytometry was performed on a LSRII flow cytometer using the FACSDiva software (Becton, Dickinson and Co.). Ten thousand to 1,000,000 events were collected per sample and analyzed using the Flowjo software.

**Differentiation of Human CD34⁺ Cells In Vitro.** BM MNCs were isolated from 12-week-old humice. CD34⁺ cells were enriched by MACs microbeads (Miltenyi Biotec). Purified cells were culture in RPMI 1640, 10% FCS at 37 °C and 5% CO₂. For the differentiation of NK cells, DCs, monocytes/macrophages, and erythrocytes, 50 ng/ml SCF, 50 ng/ml FL and 50 ng/ml IL-15; 50 ng/ml SCF, 20 ng/ml GM-CSF and 50 ng/ml IL-4; 50 ng/ml SCF and 30 ng/ml M-CSF; and 100 ng/ml SCF, 5 mg/ml IL-3 and 3 U/ml EPO were used, respectively. All of the cytokines were purchased from R&D Systems.

**NK Cell Cytotoxicity Assay and Stimulation.** Nine days after gene delivery, CD56⁺ NK cells were purified from spleen and BM by positive selection using the Stem cell PE selection Kit (Stem Cell Technologies). Cells were washed and resuspended in IMDM containing 2% FCS, and cytotoxicity against the NK-sensitive target K562 (ATCC) was determined in a 4-h lactate dehydrogenase release assay (CytoTox 96; Promega).

For in vitro stimulation, purified NK cells were cultured in RPMI 1640, 10% FCS, 2-mM L-glutamine, 1-mM sodium pyruvate, penicillin, and streptomycin, either with or without human DCs, at 37 °C and 5% CO₂ for 24 h. Human DCs were differentiated from cord blood CD34⁺ cells as described (12). Next, 50 μg/ml poly(I:C) (Sigma) was added into the culture to stimulate NK cells in vitro. For in vivo stimulation, humice were i.v. injected with 200-μg poly(I:C). IFN-γ levels in the serum or in the culture supernatants were measured with ELISA Kits (R&D Systems).

**Adenovirus Infection, ALT, and Histology.** The replication-deficient, E1 and E3-deleted, type 5 Adeno-X virus expressing green fluorescent protein (AdGFP) was purchased from Clontech. AdGFP were propagated in HEK293 cells and purified by CsCl discontinued density gradient centrifugation. Humice were challenged with 4 × 10⁶ pfu AdGFP viruses by hydrodynamic injection through the tail vein. Five days after adenovirus infection, sera were collected and analyzed for ALT activities using cobas c 111 analyzer (Roche Diagnostics Ltd.). For histological analysis, the livers were removed, embedded in paraffin and 5-μm-thick sections were prepared. The paraffin sections were stained with H&E and analyzed via a light microscope. For two-color immunofluorescence staining, after blocking of nonspecific staining, deparaffinized sections are stained with appropriate antibodies and visualized using the appropriate fluorescence filters.
were stained with optimal dilutions of PE conjugated antihuman CD56 antibody (MEM-188; Biolegend). Sections were analyzed with MIRAX MIDI Fluorescence microscope (Zeiss). Statistical Analysis. Data are presented as mean and standard error of the mean. Differences between groups were analyzed via Student t-test. A P-value of <0.05 was considered statistically significant. All calculations were performed using the Origin 8.0 software package.


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