Discussion

It had remained unknown whether antigen-specific human T cell responses can be elicited in immunodeficient mice transplanted with only human $CD34^+$ HSCs. In the present study, we analyzed the differentiation of the reconstituted human T cells and the function of the human $CD8^+$ T cells stimulated by alloantigens in hNOK mice. Because human T cells express 4 cell-surface markers (CD27, CD28, CD45RA, and CCR7) and 3 cytolytic molecules (GraA, GraB, and Per) at different levels according to their stage of differentiation and maturation [22,23,25], the analysis of phenotype and effector molecule expression in the reconstituted human T cells is useful to clarify their differentiation status and effector function. We demonstrated that the $CD27^{low}CD28^-CD45RA^{+/-}CCR7^-$ (late effector memory) and the $CD27^-CD28^-CD45RA^{+/-}CCR7^-$ (effector) subsets did not exist among the human $CD8^+$ T cells from the hNOK mice. In addition, the reconstituted human $CD8^+$ T cells did not include the $Per^{high}GraA^+GraB^+$ (effector) cells. These results suggest that the reconstituted human $CD8^+$ T cells did not have their effector function *in vivo*. On the other hand, the reconstituted human $CD8^+$ T cells included the $CD27^+CD28^+CD45RA^-CCR7^-$ (early effector memory) subsets in the hNOK mice, suggesting that the human $CD8^+$ T cells could differentiate into memory T cells in the hNOK mice in response to some stimulation. Although early effector memory $CD8^+$ T cells among the human adult $CD8^+$ T cell population include $Per^{low}GraA^+GraB^-$ cells [25], the reconstituted human $CD8^+$ T cells did not include the Per^{low}-$GraA^+GraB^-$ ones. This finding suggests that some population of early effector memory $CD8^+$ T cells in the mice were defective and could not express Per in the course of their differentiation. These results further suggest that the human $CD8^+$ T cells did not have their effector function in the hNOK mice. On the other hand, the reconstituted human $CD8^+$ T cells showed the ability to produce cytokines similar to that of human adult $CD8^+$ T cells.

The linear differentiation model supports the idea that the naive T cells differentiate directly into effector cells during the acute phase of the response and that following contraction in the numbers of the effector cells at the end of the primary response, effector memory and central memory T cells become detectable [30]. However, the human $CD8^+$ T cells reconstituted in the hNOK mice showed the lack of Per expression and $Per^{low}GraA^+$-$GraB^-$ subset, implying that the cells could not differentiate into effector cells and subsequently differentiate into memory cells. The results of phenotypic analysis and effector molecule expression of the reconstituted human $CD8^+$ T cells in the hNOK mice support the idea that the human $CD8^+$ T cells asymmetrically divided into long-lived memory cells [30,31].

A previous study demonstrated the presence of alloantigen-specific cytotoxic human T cell clones in hNOG mice [5]. However, the data from that study do not exactly reflect T cell responses *in vivo*; because alloantigen-specific human $CD8^+$ T cell clones were established from the spleen cells of the mice cultured with allogeneic target cells *in vitro*. The frequency of the human alloreactive T cell repertoire was evaluated to reflect between 0.1% and 10% of the total T cell population, whereas that of T cells specific for foreign antigen is $<1/100{,}000$ [28,29]. Since the reconstituted human $CD8^+$ T cells did not respond to alloantigen in the hNOK mice, these reconstituted human $CD8^+$ T cells may not have a foreign antigen-specific function *in vivo*. This result implies that the reconstituted human $CD8^+$ T cells did not have the appropriate T cell receptor (TCR) repertoire against foreign antigens, because the human $CD8^+$ T cells were educated by mouse MHC-peptide complexes in the thymus. CD8 co-receptors bind with a nonpolymorphic region within the $\alpha 3$ domain of class I MHC proteins, and their binding has species specificity [32–36]. Considering that human CD8 molecules fail to bind to murine MHC, the human $CD8^+$ T cells in immunodeficient mice transplanted with only human $CD34^+$ HSCs are not educated in the murine thymus.

There are several reports that antigen-specific responses are detectable in BLT mice infected with EBV or HIV [12,14,16]. Furthermore, the BLT mice produce high levels of human IgM and IgG antibodies and mediate strong immune responses *in vivo*, as demonstrated by skin xenograft rejection [13]. These results suggest that the reconstituted human T cells educated in the human thymus can have appropriate antigen-specific function in mice. A recent study demonstrated that the human $CD8^+$ T cells reconstituted in HLA transgenic NOG mice transplanted with only human $CD34^+$ HSCs expressed IFN-γ production and EBV-specific cytolytic activity in response to an EBV infection [37,38], suggesting that the reconstituted human T cells educated by human HLA class I molecules on mouse thymus cells have appropriate effector function in the mice.

In the present study, we demonstrated impaired differentiation of human $CD8^+$ T cells in the hNOK mice established with only human $CD34^+$ T cells and lack of ability to induce the effector function. Previous studies of hNOG mice showed very weak immune responses to viral antigens [8,9,11]. Thus both hNOK and hNOG mice seems to have no or weak ability in HLA-restricted T cell responses although it is unclear whether some differences exist in human T cell function between 2 humanized mice. HLA transgenic immunodeficient mice are expected to be useful for the generation of a good mouse model having human immunity.

Materials and Methods

Establishment of humanized mice

The NOK mouse strain was established by backcrossing $JAK3^{-/-}$ mice with the NOD.Cg-Prkdcscid strain for 10 generations. The NOK mice were maintained under specific pathogen-free conditions in the Center for Animal Resources and Development. Animal experiments were conducted according to the Regulation for Animal Experiments in Kumamoto University (Approval ID, C22-168: Analysis of immune responses against viral diseases by using humanized and HLA transgenic mice). Human CB was purchased from Riken Cell Bank (Tsukuba, Japan). Human $CD34^+$ cells were isolated from human CB by using a Direct CD34 Progenitor Cell Isolation Kit and an MS Column (Miltenyi Biotec, Gladbach, Germany). The usual purity of the human $CD34^+$ cells was approximately 95%. The hNOK mice were generated by injecting the isolated human $CD34^+$ cells (5×10^4 cells/mouse) into the liver of newborn NOK mice.

Blood samples

Human blood samples were taken from healthy adult individuals. Peripheral blood mononuclear cells (PBMC) were isolated from the blood by using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden).

Cell lines

721.221 cells are human B cell lines lacking HLA class I but not HLA class II [39]. 721.221 cells expressing HLA-A*2402 (.221-A*2402) and those expressing HLA-B*5201 (.221-B*5201) were generated by transfecting of HLA-A*2402 and HLA-B*5201 genes into 721.221 cells, respectively.

Flow cytometric analysis

Human leukocytes reconstituted in hNOK mice were stained with various combinations of monoclonal antibodies (mAbs): FITC-labeled anti-mouse CD45 (mCD45), PECy7-labeled human CD45 (hCD45), allophycocyanin-labeled anti-CD4, allophycocyanin, Cy7-labeled anti-CD4, AmCyan-labeled anti-CD8, Pacific blue-labeled anti-CD8, PE-labeled anti-CD19, FITC-labeled anti-Per, PE-labeled anti-GraA, Alexa647-labeled anti-GraB, PE-Cy7-labeled anti-CCR7, FITC-labeled anti-CD45RA, PE-labeled anti-CD28, allophycocyanin-Cy7-labeled anti-CD27, FITC-labeled IFN-γ and PE-Cy7-labeled TNF-α mAbs, all were purchased from BD Biosciences (San Diego, CA). ECD-labeled anti-CD3 mAb was obtained from Beckman Coulter (Fullerton, CA); and allophycocyanin-labeled IL-2 mAb, from e-bioscience (San Diego, CA). To analyze the phenotype of human T cells reconstituted in the hNOK mice, we first stained the human T cells with anti-CCR7 mAb for 30 min at room temperature, and subsequently with specific antibody against surface markers at 4°C for 30 min. The cells were washed twice with PBS containing 10% fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO). To analyze the intracellular expression of IFN-γ, Per, GraA, and GraB, we fixed cells with 4% paraformaldehyde PBS at 4°C for 20 min, and then made them permeable by incubating them at 4°C for 10 min in PBS containing 0.1% saponin (Sigma-Aldrich) and 20% FCS (permeabilizing buffer). The cells were then stained with anti-IFN-γ, anti-Per, anti-GraA, and anti-GraB mAbs at 4°C for 20 min. Finally, the stained cells were washed 3 times in the permeabilizing buffer at 4°C. The stained cells were analyzed by using a FACSCant II flow cytometer (BD Biosciences, San Jose, CA). All flow cytometric data were analyzed by using FlowJo software (Tree Star, Inc, Ashland, OR).

Cytokine production of human $CD8^+$ T cells stimulated with PMA and ionomycin

The splenocytes of hNOK mice were cultured at a density of 1×10^6 cells in 96- well plates with phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) and ionomycin (1 ng/ml). Two hours later, brefeldin A (10 ng/ml) was added to each well. After a further 4-hour incubation, the splenocytes were stained with Allophycocyanin-labeled anti-IL-2, FITC-labeled anti-IFN-γ, and PE-Cy7-labeled anti-TNF mAbs, and the stained cells were analyzed on the FACSCant II flow cytometer.

Assay for alloreactivity of human $CD8^+$ T cells

PBMC (5×10^6/mouse) from a healthy donor with HLA types HLA-A*2402/A*2402, B*5201/B*5901, and DRB1*1502/DRB1*0405 were irradiated and then intraperitoneally injected into hNOK mice. At 4 weeks after the injection, PBMC and splenocytes of the hNOK mice were harvested and analyzed for their phenotype by using the FACSCant II. To investigate IFN-γ expression of human T cells against alloantigen, we cultured the splenocytes for 6 hours at a density of 1×10^6 cells in 96-well plates with the irradiated PBMC or irradiated self-CB. Then the cultured cells were stained with anti-CD3 mAb, anti-CD8 mAb, and anti-IFN-γ mAb. For proliferation assays, carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Molecular Probes, Willow Creek Road Eugene, OR) for a final concentration of 0.5 μM was added to the cell suspension. After a 15-min incubation, the excess CFSE was removed by washing with 5% FCS/PBS; and then the cells were resuspended in RPMI1640 containing 10% FCS. The CFSE-labeled cells were cultured for 3 days with 721.221 cells expressing HLA-A*2402 or HLA-B*5201 and then were analyzed on the FACSCant II.

Statistical analysis

Results shown as bar graphs were expressed as the means ± S.E.M. One-way ANOVA followed by Dunnet's test was used for multiple comparisons. Differences were considered to be statistically significant when the p-value was less than 0.05.

Supporting Information

Figure S1 Phenotypic analysis of reconstituted human $CD8^+$ T cells stimulated with alloantigen in hNOK mice hNOK mice were immunized for 31 days with irradiated human PBMC from a healthy donor with HLA-A*2402/A*2402, HLA-B*5201/B*5901, and HLA-DRB1*1502/DRB1*0405. Then the phenotype of human $CD8^+$ T cells among PBMC from the hNOK mice was analyzed on 0, 7, and 31 days after the immunization. Splenocytes from the same mice were examined at day 31. (A) Representative results of 5-color flow cytometric analysis of CCR7CD45RACD27CD28 subsets in human $CD8^+$ T cell population of PBMC and splenocytes are shown. (B) Representative results for Per, GraA, and GraB expression by the human $CD8^+$ T cells among splenocytes from the hNOK mice are shown.
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Author Contributions

Conceived and designed the experiments: MT. Performed the experiments: YS HT NK SN. Analyzed the data: YS HT NK TU. Contributed reagents/materials/analysis tools: NN. Wrote the paper: MT.

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