厚生労働科学研究費補助金

エイズ対策研究事業

HIV 感染モデルマウスの樹立および HIV 特異的細胞傷害性 T 細胞によるエイズ発症遅延機序の解析

平成22年度　総括研究報告書

研究代表者　佐藤　義則

平成23年（2011年）3月

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I. 総括研究報告

厚生労働科学研究費補助金(エイズ対策研究事業)
総括研究報告書

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研究代表者: 佐藤 義則(熊本大学エイズ学研究センター COEリサーチ・アソシエイト)

研究要旨

HIV感染に対する免疫応答の解析が困難である理由のひとつとして、小動物を用いたHIV感染実験系が確立されていないことが挙げられる。 そこで我々はヒト免疫系をマウス生体内に構築したヒト免疫構築マウス(ヒト化マウス)に着目し、ヒト化マウスを用いた HIV 感染実験系の確立を目指した。しかしながら、高度免疫不全マウスにヒト幹細胞を移植したヒト化マウスでは、ヒトT細胞が末梢血や脾臓で確認されているものの、エフェクター機能を持たないことやアロ抗原刺激に対する反応が起きないことから、更なる改良を加えたヒト化マウスの作製が必要となった。

我々はヒト CD8T 細胞の機能が誘導できると期待される HLA-B*51:01 遺伝子を導入したNOK/B51 トランスジェニックマウス(NOK/B51Tg)を新たに樹立し、このマウスにヒト $CD34^+$細胞を移植してヒト化 NOK/B51Tg マウスを作製した。 このヒト化 NOK/B51Tg マウスは、ヒト化 NOK マウスと同様に、ヒト $CD34^+$細胞を移植後 10 週目からヒト CD4、CD8T 細胞の再構築が確認できた。 このヒト化NOK/B51Tg マウスに HIV-1(NL43 株)を腹腔内投与で感染させ、ヒトT細胞の割合を解析した結果、ヒト CD4T 細胞の割合は感染 2 週間目以降から非感染郡に比べ徐々に減少した。 血漿中のHIV-RNA 量を調べた結果、感染 14～28 日目から検出ができ、感染 42 日目においても検出することができた。 さらに感染 42 日目の血漿中の HIV-RNA のゲノム全塩基配列を解析した結果、NOK/B51Tg マウスの 7 匹中 4 匹からゲノム塩基配列に遺伝子変異が見つかり、その変異は pol, vpu, env 遺伝子領域内で見つかった。 以上の結果から、我々が作成したヒト化 NOK/B51Tg マウスではHIV 感染者に見られるような HIV ゲノム塩基配列の遺伝子変異が見つかり、ヒト化 NOK/B51Tg マウス内で再構成された細胞傷害性T細胞の免疫圧による逃避変異ウイルスの選択(HIV に対する免疫応答)が起きた可能性が考えられる。

A. 研究目的

長期にわたってHIVの増殖を抑えるためには、HIV 特異的細胞傷害性 T 細胞によるウイルス感染細胞の排除が重要であることが知られている。当研究室においても、長期にわたってエイズを発症しないHIV感染者から非常に強いHIV増殖抑

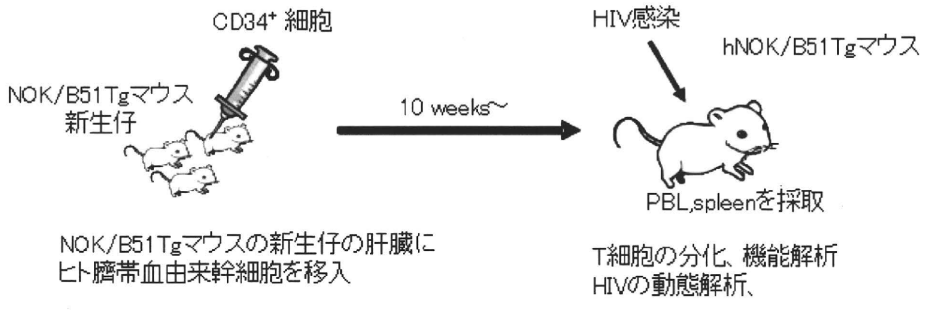
制能を示す細胞傷害性T細胞(CTL)の単離に成功し、CTLがウイルス感染細胞の排除に重要であることを報告している。 一方で、長期 HIV 感染における免疫応答の解析が困難である理由のひとつとして、小動物を用いた HIV 感染実験系が確立されていない点が挙げられる。 そこで我々は、新たに高度免疫不全マウス(NOK マウス)を用いたヒト化マウスを作製し、ヒト化マウスの基礎知見を得るため、発生したヒトT細胞の分化・機能を解析した。 その結果、ヒト化NOKマウス内で再構成されたヒト CD8T細胞は、細胞傷害活性を示すエフェクター表現型の集団がほとんど含まれず、パーフォリンの発現がヒト PBMC 中に含まれるヒト CD8T 細胞に比べ低いということを明らかにした。さらに、アロ抗原となるヒト PBMC で免疫したヒト化 NOK マウスの脾細胞を *in vitro* で再刺激を加えてもヒト CD8T 細胞の IFN-γ 産生および細胞増殖を誘導できないことを前年度の解析で明らかにした。

そこで本年度は、ヒト化マウス内でヒトT細胞の分化・機能を誘導できると期待できる、HLA-B*51:01 を導入したヒト化 NOK/B51Tg マウスの樹立を試みた。 また、そのヒト化NOK/B51Tgマウスの長期HIV感染における免疫応答を検討するため、ヒト化NOK/B51Tgマウスにおける HIV 感染系の確立、さらに HIV 感染マウスにおけるHIVの動態を解析するため、HIV感染ヒト化マウスから回収した HIV のゲノム塩基配列を解析した。 今回の研究で作製した HLA 発現ヒト化マウスでは、ヒトの造血・免疫系を確立した際に問題となる胸腺での適切な発生・分化が誘導でき、これまでのモデルマウスより成熟したヒト免疫系の構築が可能であると考える。 また、CTLの免疫圧から逃避するHIVの逃避メカニズム等を解析できるモデルとなる点が優れており、これらの研究を通じて、HIV 感染に対する免疫応答の解明と新規エイズ治療法の開発に大きく貢献できることが期待できる。

B. 研究方法

NOK/B51Tgマウスは、NOD/SCID/B51Tgマウスと NOK マウスの掛け合わせにより樹立した。臍帯血単核球からMACSを用いてヒト CD34⁺ 細胞を分離し、NOK/B51Tg マウスの新生仔の肝臓へ移植した。 移植10週後、ヒト T 細胞の生着を調べるためにマウスの尾静脈から末梢血を経時的に採取した(図1)。 ヒトT細胞の生着が確認できたマウスは、HIV-1(NL43 株)を腹腔から感染させ、感染が確認されたマウスのヒトT細胞の系時的な割合を、フローサイトメトリーを用いて解析し

図1 方法



た。 また、系時的に末梢血から血漿を分離し、そこに含まれるHIV-1 ゲノム塩基配列を解析した。

(倫理面への配慮)

本研究におけるヒト由来の検体の使用と遺伝子解析、および理化学研究所(理研)から購入した臍帯血単核球は提供者に対して既にインフォームド・コンセントを行い得たものであり、本研究によって新たに提供者に危険を及ぼすことは無く、当大学の倫理審査委員会にて承認済みである。 また、動物実験についても既に当大学の動物実験委員会にて承認済みである。

C. 研究結果

NOK/B51Tg マウスは、NOD/SCID/B51Tg マウスと NOK マウスとの掛け合わせにより樹立した。また NOK/B51Tg マウスの脾細胞を回収し、HLA-B5101 の発現を確認した(図 2)。 ヒト $CD34^+$ 細胞を移植した NOK/B51Tg マウスでは、移植後10週目の末梢血中にヒトT細胞であるヒトCD4T細胞およびヒト CD8T 細胞のそれぞれが確認できた(図3A)。 ヒト化 NOK/B51Tg マウスにHIV-1(NL432)を感染させると、2 週間後からヒトCD4T細胞の割合が減少した(図3A および3B)。

HIV-1 感染ヒト化 NOK/B51Tg マウスの血漿を系時的に採取し、HIV-RNA の検出を行った。 血漿中の HIV-RNA は、感染後2週間または4週間で検出できるようになり、ヒト化 NOK/B51Tg マウスに HIV-1 が感染することが確認できた(図4)。 さらに、HIV 感染 6 週間後のヒト化 NOK/B51Tg マウスの血漿中からHIV-RNA を分離し、ゲノム塩基配列を解析した。 7匹の HIV 感染ヒト化 NOK/B51Tg マウスから分離したHIV-RNA では、そのうち4匹から HIV-RNA の遺伝子変異が見つかった(図5)。 一方、NOK マウス(B51-)では感染した HIV-1 のゲノム塩基配列に遺伝子変異は見つからなかった(図5)。

D. 考察

小動物を用いた HIV 感染実験系の確立は、HIV 感染における経時的な免疫応答や治療効果を解析するツールとなるとともに、飼育に大掛かりな施設、設備、高額の飼育費を必要とするサルやチンパンジーにかわる代替動物として期待され、ヒト化マウスを用いた研究は既に国内外で始まっている。 しかしながら、これまでに報告されている高度免疫不全マウスにヒト幹細胞を移植したヒト化マウスでは、ヒト T 細胞が末梢血や脾臓で確認されているものの、エフェクター機能を持たないことやアロ抗原刺激に対する反応が起きないことから、更なる改良を加えたヒト化マウスの作製が必要となった。 通常、T 細胞が機能を有するためには、TCRとCD8またはCD4 コレセプターによってクラスIまたはクラス II MHC-ペプチド複合体との相互作用を必要とする。 CD8 とクラス I MHC の $\alpha 3$ ドメインとの相互作用は種特異性を示し、ヒト TCR とマウス MHC の相互作用の親和性がヒト TCR とヒト MHC のそれと大きく異なる。 また近年の研究から、末梢においても CD8 と MHC の相互作用が CD8T 細胞のエフェクター機能の誘導に大きく関係することも明らかとなっている。 そこで我々は、NOK マウスに HLA-B*51:01 を組み込んだ NOK/B51Tg マウスの樹立を行い、このマウスでヒト化 NOK/B51Tg を作製し HIV-1 を感染させた。その結果、ヒト化 NOK/B51Tg マウスはヒト CD4T

細胞の減少やHIV-RNAの検出が可能であり、ヒトのHIV感染初期症状と類似したin vivo モデルとなり得ることが期待できた。

さらに、HIV感染6週間目のヒト化NOK/B51Tgマウスの血漿中からHIV-RNAを分離しゲノム塩基配列を調べた結果、遺伝子変異を見つけることが出来た。またHIV-1のゲノム塩基配列の系時的な解析から、遺伝子変異は早い箇所で感染2週目から確認できた。HIV感染者のHIVゲノム塩基配列の解析では、感染2週目から変異が誘導されている報告もあり、ヒト化NOK/B51Tgマウスは HIVの逃避変異体を解析するツールとなることも期待できる。

一般的にHIVが変異する要因として、中和抗体やCTLによる免疫圧からの逃避が考えられる。今回解析したヒト化NOK/B51Tgマウス内で変異したHIV-1のアミノ酸部位のいくつかは、既知のエピトープとして存在する部位であった。またHIV感染2週目から変異が誘導されたことを考えると、ヒト化NOK/B51Tgマウス内で再構築されたヒトT細胞のCTLによる免疫圧から逃避したウイルスが出現したと推測でき、今後はHIV特異的CTLが誘導されたかについて詳細な解析を行う必要がある。

E. 結論

今回の研究で我々は、ヒト幹細胞を移植して作製したヒト化NOK/B51TgマウスにHIVを感染させると、HIV感染者と類似したヒトT細胞の減少とHIVの遺伝子変異を誘導することに成功した。近年の報告では、HLA発現ヒト化マウスのヒトCD8 T細胞はEBV感染において抗原特異的細胞傷害活性が確認されており、HIV感染においてもヒトCD8 T細胞のHIVに対する抗原特異的細胞傷害活性を今後、解析していきたい。ヒト化マウスモデルはさらに改良を加えることにより、ヒト免疫応答を解析できる小動物モデルとして有用なツールとなり得ると考えられ、ウイルス感染症の病因や予防、治療の研究に大きく貢献することができると期待する。

F. 健康危険情報

なし。

G. 研究発表

1）論文発表

Sato Y, Takata H, Kobayashi N, Nagata S, Nakagata N, Ueno T, Takiguchi M. Failure of effector function of human CD8$^+$ T cells in NOD/SCID/JAK3$^{-/-}$ immunodeficient mice transplanted with human CD34$^+$ hematopoietic stem cells. *PLoS ONE.* 5; e13109, 2010.

1）学会発表

Sato Y, Takata H, Nagata S, Takiguchi M. Failure of effector function of human CD8$^+$ T cells in NOD/SCID/JAK3$^{-/-}$ immunodeficient mice transplanted with human CD34+ hematopoietic stem cells. 11th Kumamoto AIDS Seminar -GCOE Joint International Symposium, 2010, Kumamoto. (ポスター発表)

H. 知的財産権の出願・登録状況（予定を含む。）

なし。

図2

NOK/B51Tgマウスの樹立

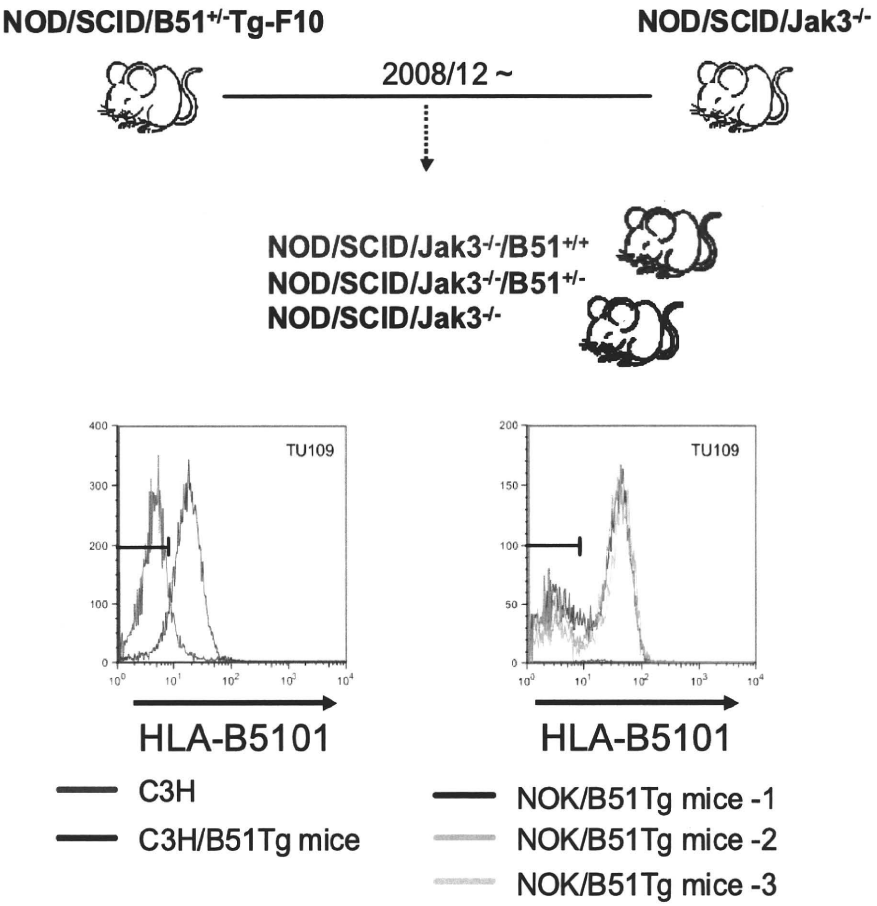
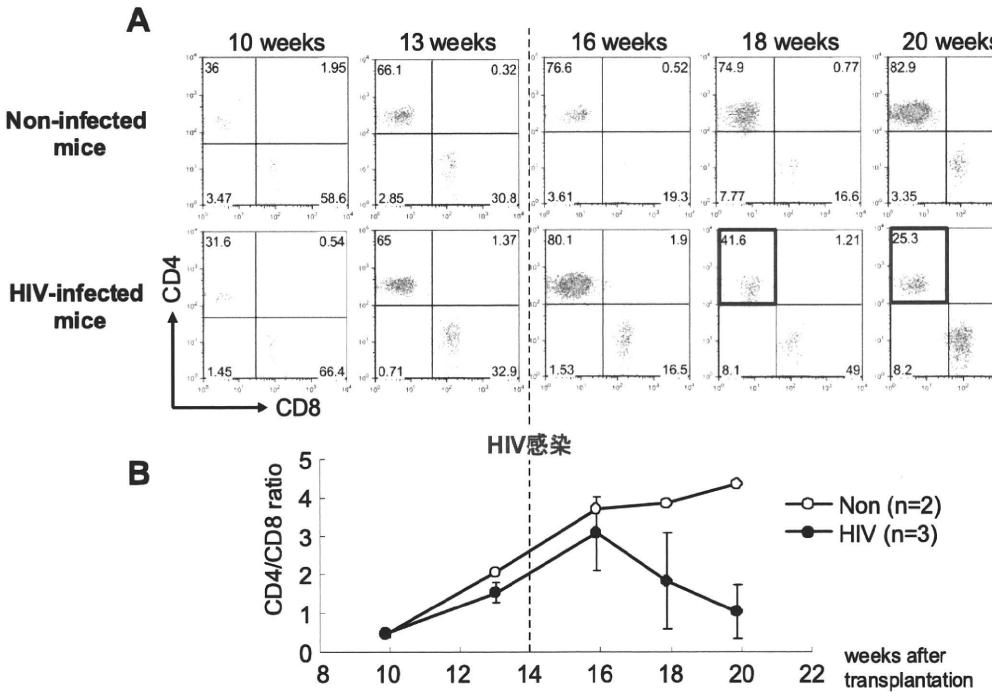


図3

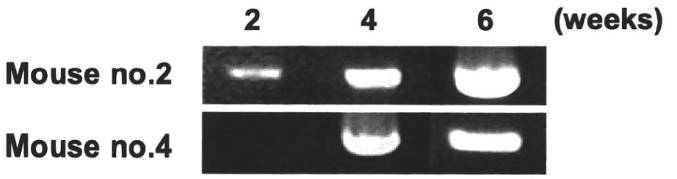
HIV感染hNOK/B51Tgマウスのヒト T細胞の割合の系時的な変化



HIV(NL432)を感染させたhNOK/B51Tgマウスでは、ヒトCD4T細胞の割合が感染2週目以降で減少した。

図4

HIV感染hNOK/B51Tgマウスの血漿中に含まれるHIV-RNAの検出



HIV感染hNOK/B51Tgマウスの血漿中では、感染2～4週目以降にHIV-RNA (NL432)が検出された。

図5

HIV感染hNOK/B51Tgマウスの血漿中から分離したHIVゲノム塩基配列の解析

Experimental Group	Number of mice		
	Total	No mutation	Mutation
B51(+/-)	7	3	4
B51(-/-)	1	1	0

HIV感染hNOK/B51Tgマウスの血漿中から分離したHIV(NL432)では、ゲノム塩基配列にいくつかの変異が見られた。

II. 研究成果の刊行に関する一覧表

佐藤義則

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
佐藤義則、高田比呂志、小林直樹、永田紗矢香、中潟直己、上野貴将、滝口雅文	Failure of effector function of human $CD8^+$ T cells in NOD/SCID/$JAK3^{-/-}$ immunodeficient mice transplanted with human $CD34^+$ hematopoietic stem cells.	*PLoS ONE*	5	e13109	2010

III. 研究成果の刊行物・別刷

OPEN ACCESS Freely available online

PLoS one

Failure of Effector Function of Human CD8$^+$ T Cells in NOD/SCID/JAK3$^{-/-}$ Immunodeficient Mice Transplanted with Human CD34$^+$ Hematopoietic Stem Cells

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Abstract

Humanized mice, which are generated by transplanting human CD34$^+$ hematopoietic stem cells into immunodeficient mice, are expected to be useful for the research on human immune responses. It is reported that antigen-specific T cell responses occur in immunodeficient mice transplanted with both human fetal thymus/liver tissues and CD34$^+$ fetal cells, but it remains unclear whether antigen-specific T cell responses occur in those transplanted with only human CD34$^+$ hematopoietic stem cells (HSCs). Here we investigated the differentiation and function of human CD8$^+$ T cells reconstituted in NOD/SCID/Jak3$^{-/-}$ mice transplanted with human CD34$^+$ HSCs (hNOK mice). Multicolor flow cytometric analysis demonstrated that human CD8$^+$ T cells generated from the CD34$^+$ HSCs comprised only 3 subtypes, i.e., CD27^{high}CD28$^+$CD45RA$^+$CCR7$^+$, CD27$^+$CD28$^+$CD45RA$^-$CCR7$^+$, and CD27$^+$CD28$^+$CD45RA$^-$CCR7$^-$ and had 3 phenotypes for 3 lytic molecules, i.e., perforin(Per)$^-$granzymeA(GraA)$^-$granzymeB(GraB)$^-$, Per$^-$GraA$^+$GraB$^-$, and Per^{low}GraA$^+$GraB$^+$. These CD8$^+$ T cells failed to produce IFN-γ and to proliferate after stimulation with alloantigens. These results indicate that the antigen-specific T cell response cannot be elicited in mice transplanted with only human CD34$^+$ HSCs, because the T cells fail to develop normally in such mice.

Citation: Sato Y, Takata H, Kobayashi N, Nagata S, Nakagata N, et al. (2010) Failure of Effector Function of Human CD8$^+$ T Cells in NOD/SCID/JAK3$^{-/-}$ Immunodeficient Mice Transplanted with Human CD34$^+$ Hematopoietic Stem Cells. PLoS ONE 5(10): e13109. doi:10.1371/journal.pone.0013109

Editor: Derya Unutmaz, New York University, United States of America

Received July 13, 2010; **Accepted** September 9, 2010; **Published** October 1, 2010

Funding: This research was supported by grant-in-aid for scientific research from the Ministry of Health to YS (No. 08062169), by the grant to MT (No. 20390134) and that from the Global COE program "Global Education and Research Center Aiming at the control of AIDS", launched as a project commissioned by the Ministry of Education, Science, Sports, and Culture, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Humanized mice are generated by transplanting human CD34$^+$ hematopoietic stem cells (HSCs) into immunodeficient mice and are expected to become a useful tool in studies on human T cell immune responses, infectious diseases, preclinical testing of vaccines, and new therapeutic strategies. Previous studies showed long-term human T cell and B cell reconstitution in NOD/SCID/γc^{null} immunodeficient mice transplanted with human CD34$^+$ HSCs (hNOG mice) [1–3]. Human IgM, IgG, and IgA were detectable in the serum of these mice; and class-switching of immunoglobulin in human cord blood (CB)-derived B cells properly occurred in the mice [4–7], indicating that the human B cells can develop from human CD34$^+$ HSCs in the hNOG mice and are functionally competent to produce immunoglobulins in them. Furthermore, previous studies demonstrated that human CD4/CD8 double-positive and human CD4/CD8 single positive T cells were observed in the thymus of the hNOG mice and that the latter were found in the spleen and peripheral blood of these animals [3–5,7,8]. These human T cells expressed predominantly αβ T cell receptors in the thymus and the spleen of the recipient, whereas CD45RA$^+$ naive T cells were identified in the spleen and the peripheral blood of the hNOG mice [4,9,10]. These results suggest the possibility that the human T cells respond to highly diverse molecules. Additionally, proliferation and IFN-γ expression of EBV-specific human CD8$^+$ T cells have been demonstrated in hNOG mice and in Rag2$^{-/-}$γc$^{-/-}$ mice transplanted with human CD34$^+$ HSCs after an EBV infection [8,11]. In contrast, high-dose injection of EBV caused a fatal lymphoproliferative disorder in the hNOG mice, whereas lower-dose injection induces an apparently asymptomatic persistent infection [11]. These findings suggest that the human T cell responses were not able to control the replication of EBV in the hNOG mice.

On the other hand, antigen-specific T cell immune responses had definitely been shown in humanized NOD/SCID mice established by transplanting human fetal thymus/liver tissues and CD34$^+$ fetal liver cells into them (BLT mice) [12–16]. However, there are no studies providing reliable evidence as to whether or not antigen-specific T cell responses are induced in humanized mice established by transplanting only human CD34$^+$ HSCs into immunodeficient mice.

The phenotypic analysis of human T cells in humanized mice is useful to clarify their differentiation and effector function, because the phenotypic classification of human T cells reflects their differentiation and effector function. Previous studies demonstrated that human CD8$^+$ T cells change the expression levels of co-

stimulatory molecules (CD27, CD28, and CD45RA) [17–19] and chemokine receptor CCR7 on their surface according to their differentiation and maturation [20,21]. The phenotypic analysis of human $CD8^+$ T cells showed that $CD27^{high}CD28^+CD45RA^+CCR7^+$, $CD27^+CD28^+CD45RA^-CCR7^+$, $CD27^+CD28^+CD45RA^-CCR7^-$, $CD27^{low}CD28^-CD45RA^{+/-}CCR7^-$, and $CD27^-CD28^-CD45RA^{+/-}CCR7^-$ have characteristics of naive, central memory, early effector memory, late effector memory, and effector $CD8^+$ T cells, respectively [22,23]. Moreover, human $CD8^+$ T cells express 3 key cytolytic effector molecules, i.e., perforin (Per), granzyme A (GraA), and granzyme B (GraB) in response to their differentiation [24]. Five subpopulations of human $CD8^+$ T cells defined by these cytolytic molecules exist and appear sequentially during $CD8^+$ T cell differentiation: $Per^-GraA^-GraB^-$, $Per^-GraA^+GraB^-$, $Per^{low}GraA^+GraB^-$, $Per^{low}GraA^+GraB^+$, and $Per^{high}GraA^+GraB^+$ [25]. Thus, the functional subsets of human $CD8^+$ T cells can be identified by the phenotypic classification and the expression of these 3 cytolytic molecules [25]. By using these classifications of human $CD8^+$ T cells, the differentiation and function of human $CD8^+$ T cells reconstituted in humanized mice can be clarified in detail. A previous study showed that the $CD45RA^+CCR7^-$ subset were detected in hNOG mice [10], suggesting the possibility that human $CD8^+$ T cells can be differentiated in the mice. However, since this study did not demonstrated the existence of effector subset, $CD27^-CD28^-CD45RA^{+/-}CCR7^-$, it still remains unclear if human $CD8^+$ T cells can be differentiated into effector cells in hNOG mice.

In the present study, to clarify antigen-specific human $CD8^+$ T cell responses in immunodeficient mice transplanted with only human $CD34^+$ HSCs, we established NOD/SCID/$JAK3^{-/-}$ (NOK) mice transplanted with human $CD34^+$ HSCs (hNOK mice) and investigated the differentiation and function of human $CD8^+$ T cells reconstituted in these mice. Especially, we focused on performing phenotypic classification based on the expression of the above-mentioned effector molecules and the alloreactivity of human $CD8^+$ T cells reconstituted in the mice.

Results

Reconstitution of human T cells in hNOK mice

hNOK mice were established by transplanting human $CD34^+$ HSCs isolated from human CB into the liver of newborn NOK mice. In order to investigate the reconstitution of the human immune system in these mice, we obtained PBMC from the mice at 10, 12 and 17 weeks after the transplantation and then analyzed them by using flow cytometry for detecting human T and B cells. As shown in Table 1, the cells expressing common human leukocyte antigen CD45 ($hCD45^+$) were found in all hNOK mice at 10 weeks after the transplantation (n = 31). Among the $hCD45^+$ cells, human B cells, identified by the expression of CD19, were observed in all hNOK mice, whereas human T cells, identified by the expression of CD3, were observed in approximately 60% of the mice (Table 1). In the mice carrying human T cells, the proportion of human T cells gradually increased from 10 to 17 weeks after the transplantation and reached a level at 17 weeks similar to the proportion of $CD8^+$ T cells in the human adult PBMC population (Figure 1A and 1B). The human T cells obtained during the period of 10-17 weeks post transplantation included both human $CD4^+$ and $CD8^+$ T cells (Figure 1C), though the ratio of human CD4/CD8 T cells gradually increased from the 10 to 17 weeks (Figure 1D). These results show that human $CD8^+$ and $CD4^+$ T cells were generated and maintained in the mice.

Phenotypic analysis of human T cells reconstituted in hNOK mice

Human peripheral $CD8^+$ T cells are classified into the following 5 major populations based on their expression of 4 cell-surface

Table 1. Proportion of human immune cells in hNOK mice.

		% nucleated cells					% nucleated cells		
Cord blood			**hCD45 gated**		**Cord blood**			**hCD45 gated**	
Mouse No.		**$hCD45^+$**	**$CD3^+$**	**$CD19^+$**	**Mouse No.**		**$hCD45^+$**	**$CD3^+$**	**$CD19^+$**
Donor 1	1	12.8	0.0	54.6	Donor 4	1	62.1	46.7	48.3
	2	32.7	32.3	61.8		2	37.4	37.0	58.8
	3	55.1	55.7	40.3		3	57.4	23.3	67.8
	4	59.1	0.0	89.3		4	47.3	45.0	49.2
	5	5.1	0.5	94.9		5	21.4	0.0	91.0
	6	34.2	38.0	57.5		6	37.1	33.7	57.5
Donor 2	1	45.8	0.1	67.9	Donor 5	1	32.1	0.2	95.0
	2	35.1	13.1	57.9		2	10.3	0.0	92.9
	3	55.6	49.7	40.6		3	64.8	30.2	67.3
	4	19.3	0.0	65.5		4	44.2	0.0	96.5
	5	69.4	39.5	48.4		5	3.5	0.0	81.0
Donor 3	1	5.8	0.4	76.2	Donor 6	1	6.6	5.2	81.0
	2	18.9	47.0	43.4		2	7.7	81.3	13.2
	3	37.6	84.1	11.7	Donor 7	1	10.5	34.1	60.7
	4	10.7	0.2	79.7		2	50.3	42.2	54.0
	5	58.4	73.6	22.6					

For generation of hNOK mice, human $CD34^+$ cells derived from 7 cord blood samples were transplanted into newborn NOK mice. PBMC of the hNOK mice were analyzed for the engraftment of human immune cells at 10 weeks after the transplantation (n = 31).

doi:10.1371/journal.pone.0013109.t001

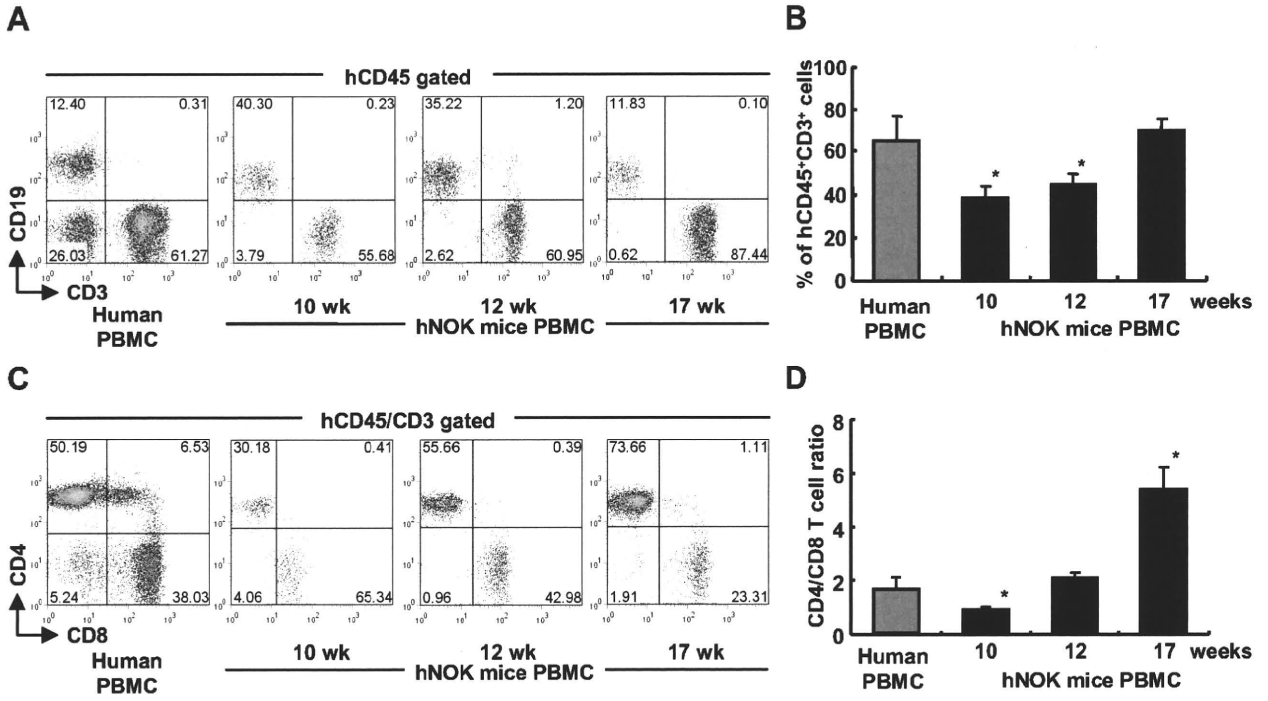

Figure 1. Flow cytometric analysis of human T cells reconstituted in hNOK mice. (A) A representative result for hCD45$^+$CD3$^+$ T cells and hCD45$^+$CD19$^+$ B cells among PBMC from hNOK mice at 10, 12, and 17 weeks after the transplantation. (B) Summarized results showing hCD45$^+$CD3$^+$ T cell proportion in PBMC from hNOK mice (n = 20) at 10, 12 and 17 weeks. (C) A representative result for human CD4$^+$ and CD8$^+$ T cell proportions in PBMC from hNOK at 10, 12 and 17 weeks after the transplantation. (D) Summarized results showing human CD4/CD8 T cell ratio for PBMC from hNOK mice at 10, 12, and 17 weeks. Human PBMC from healthy adult individuals (n = 4) are shown as a control. Bar graph data are shown as the mean ± SEM of 7 independent experiments. *, $p<0.05$, human PBMC vs. hNOK mouse PBMC.
doi:10.1371/journal.pone.0013109.g001

markers: CD27^{high}CD28$^+$CD45RA$^+$CCR7$^+$ (naive subset), CD27$^+$CD28$^+$CD45RA$^-$CCR7$^+$ (central memory subset), CD27$^+$CD28$^+$CD45RA$^-$CCR7$^-$ (early effector memory subset), CD27^{low}CD28$^-$CD45RA$^{+/-}$CCR7$^-$ (late effector memory subset), and CD27$^-$CD28$^-$CD45RA$^{+/-}$CCR7$^-$ (effector subset) [22,23]; whereas human peripheral CD4$^+$ T cells are also classified into 5 major populations by the same 4 cell-surface markers, i.e., CD27$^+$CD28$^+$CD45RA$^+$CCR7$^+$ (naive subset), CD27$^+$CD28$^+$CD45RA$^-$CCR7$^+$ (central memory subset), CD27$^+$CD28$^+$CD45RA$^-$CCR7$^-$ (Th0 effector memory subset), CD27$^-$CD28$^+$CD45RA$^-$CCR7$^-$ (Th1/2 effector memory subset), and CD27$^-$CD28$^-$CD45RA$^-$CCR7$^-$ (effector subset) [26]. To examine the differentiation of human T cells reconstituted in the hNOK mice, we analyzed the phenotype of human T cells among PBMC from the mice at 17 weeks after the transplantation and that of T cells among PBMC from adult humans for comparison. Representative results for an individual hNOK mouse and an adult human are shown in Figure 2A; and a summary of the findings, in Figure 2B. The reconstituted human CD8$^+$ T cell population included naive (39.7±26.9%), central memory (18.9±9.7%), and early effector memory (18.4±15.9%) subsets. Late effector memory (0.9±1.6%) and effector (0.8±2.6%) subsets were hardly detected in the mice. These results suggest that the human CD8$^+$ T cells did not differentiate into late effector or effector human T cells in the mice.

The phenotypic analysis of the reconstituted human CD4$^+$ T cell population in the PBMC from the mice revealed that it included naive (27.5±23.0%), central memory (31.2±10.1%), Th0 effector memory (28.1±18.8%), and Th1/2 effector memory (9.0±11.4%) subsets. The effector (0.06±0.15%) subset was hardly detected, suggesting that the human CD4$^+$ T cells did not differentiate into effector T cells in these mice.

Expression of 3 cytolytic effector molecules in reconstituted human CD8$^+$ T cells

Human peripheral CD8$^+$ T cells are classified into the following 5 major populations based on their expression levels of 3 effector molecules: Per$^-$GraA$^-$GraB$^-$, Per$^-$GraA$^+$GraB$^-$, Per^{low}GraA$^+$GraB$^-$, Per^{low}GraA$^+$GraB$^+$, and Per^{high}GraA$^+$GraB$^+$ [25]. To investigate the effector function of the human CD8$^+$ T cells reconstituted in the hNOK mice, we examined the expression of these effector molecules in human CD8$^+$ T cells among splenocytes from the mice. A representative result and summary of the analysis for 6 hNOK mice as well as for PBMC from 3 adult humans are shown in Figure 3A and 3B, respectively. The human CD8$^+$ T cells included Per$^-$GraA$^-$GraB$^-$, Per$^-$GraA$^+$GraB$^-$, and Per^{low}GraA$^+$GraB$^+$ cells. But they did not include Per^{low}GraA$^+$GraB$^-$ or Per^{high}GraA$^+$GraB$^+$ ones, though the latter were found among human adult PBMC. These results indicate that the reconstituted human CD8$^+$ T cells did not have effector function in the mice and that the human CD8$^+$ T cells did not differentiate into effector cells in the mice.

Ability of reconstituted human CD8$^+$ T cells to produce cytokines

Human CD8$^+$ T cells producing IL-2 but not IFN-γ are mainly found in naive and central memory CD8$^+$ T cell populations, whereas IFN-γ and TNF-α are mainly produced by effector memory and effector CD8$^+$ T cells among human PBMC [22,27].

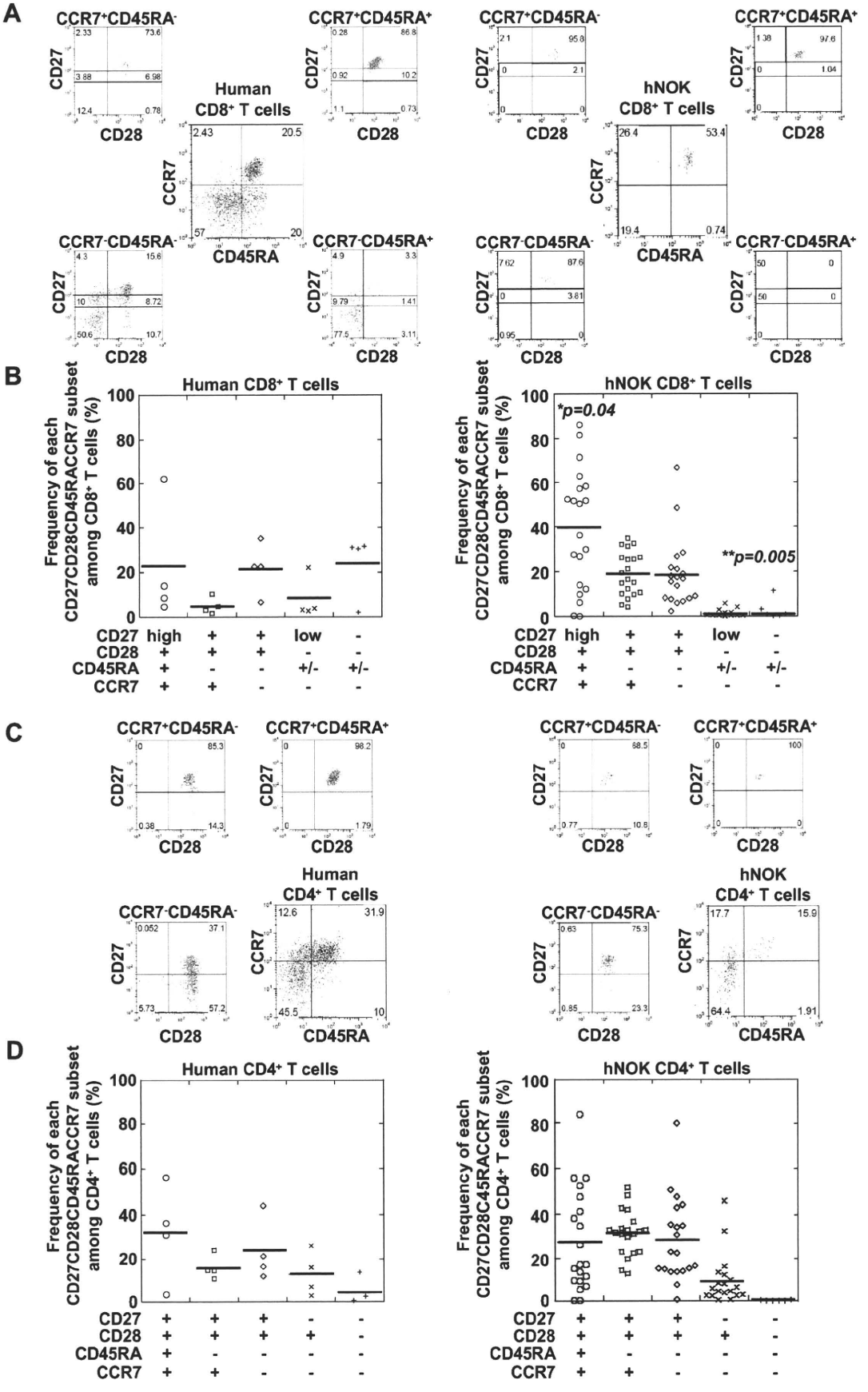
A
CCR7+CD45RA-
CCR7+CD45RA+
CCR7-CD45RA-
CCR7-CD45RA+
Human CD8+ T cells
hNOK CD8+ T cells
CD27
CD28
CCR7
CD45RA
B
Human CD8+ T cells
hNOK CD8+ T cells
Frequency of each CD27CD28CD45RACCR7 subset among CD8+ T cells (%)
*p=0.04
**p=0.005
CD27 high + + low -
CD28 + + - - -
CD45RA + - - +/- +/-
CCR7 + + - - -
C
Human CD4+ T cells
hNOK CD4+ T cells
D
Human CD4+ T cells
hNOK CD4+ T cells
Frequency of each CD27CD28CD45RACCR7 subset among CD4+ T cells (%)

Figure 2. Phenotypic classification of human CD8$^+$ T cells reconstituted in hNOK mice. Human T cells among PBMC from hNOK mice at 17 weeks after the transplantation were analyzed for the expression of the following cell-surface markers: CD4, CD8, CD27, CD28, CD45RA, and CCR7. (A) A representative result of 5-color flow cytometric analysis of the human CD8$^+$ T cell population among PBMC from a hNOK mouse (right data) and an adult human (left data). (B) Summarized results showing frequency of subsets among the human CD8$^+$ T cells isolated from the PBMC from hNOK mice (n = 20, right data) and human adult individuals (n = 4, left data). (C) A representative result of 5-color flow cytometric analysis of human CD4$^+$ T cell population in PBMC from hNOK mice (right data) and a human adult individual (left data). (D) Summarized result showing human CD4$^+$ T cell proportion in PBMC from hNOK mice (n = 20, right data) and human adult individuals (n = 4, left data). Each symbol represents 1 mouse; the mean value is shown as a horizontal solid line. *, $p<0.05$; **, $p<0.01$, human PBMC vs. hNOK mice PBMC.
doi:10.1371/journal.pone.0013109.g002

To investigate the ability of the human CD8$^+$ T cells reconstituted in the hNOK mice to produce these cytokines, we stimulated splenocytes isolated from these mice with PMA and ionomycin *in vitro* and measured the production of the above cytokines. A representative result and summary of the analysis for 12 hNOK mice are given in Figure 4A and 4B, respectively. The human CD8$^+$ T cells reconstituted in the mice produced IFN-γ, TNF-α, and IL-2 (Figure 4A). The frequency of IFN-γ-producing CD8$^+$ T cells positively correlated with that of the effector memory (CD27$^+$CD28$^+$CD45RA$^-$CCR7$^-$ plus CD27^{low}CD28$^-$CD45RA$^{+/-}$CCR7$^-$) subset, whereas the frequency of IL-2$^+$IFN-γ$^-$TNF-α$^-$-producing CD8$^+$ T cells negatively correlated with that of effector memory subset (Figure 4B). These results indicate that the human CD8$^+$ T cells had the ability to produce these cytokines in the mice.

Alloreactivity of human CD8$^+$ T cells reconstituted in hNOK mice

It is well known that 0.1–10% of human T-cell repertoire react with alloantigens compared with a frequency of <1/100,000 for foreign antigens in humans [28,29]. We investigated alloreactivity of human CD8$^+$ T cells reconstituted in hNOK mice to clarify the responsiveness of antigen-specific human CD8$^+$ T cells. We immunized hNOK mice with irradiated human PBMC from a healthy donor with HLA-A*2402/A*2402, B*5201/B*5901, and DRB1*1502/DRB1*0405 (allo-PBMC) (n = 4) and analyzed the phenotype of human CD8$^+$ T cells reconstituted in the mice on 7 days or 31 days after the PBMC injection. Flow cytometric analysis demonstrated that CD27$^-$CD28$^-$CD45RA$^{+/-}$CCR7$^-$ effector CD8$^+$ T cell subsets and Per^{high}GraA$^+$GraB$^+$ cells were not induced in the mice on 7 days and 31 days after the PBMC injection (Figures S1A and S1B). We further analyzed the ability of the human CD8$^+$ T cells to produce IFN-γ after allo-PBMC stimulation. The splenocytes from the mice obtained at 31 days after injection of the irradiated PBMC into the mice were cultured with the irradiated PBMC *in vitro* for 7 days, and then the ability of the human CD8$^+$ T cells to produce IFN-γ was measured after stimulation with the irradiated PBMC. As shown in Figure 5A, the human CD8$^+$ T cells and the CD8$^-$ T cells (mostly CD4$^+$ T cells) reconstituted in the mice did not produce IFN-γ, suggesting that both CD8$^+$ T cells and CD4$^+$ T cells could not recognize alloantigen. In addition, we analyzed the ability of the human CD8$^+$ T cells to proliferate after stimulation with alloantigens. The splenocytes of the mice on 31 days were labeled with CFSE (carboxyfluorescein diacetate N-succinimidyl ester); and the cells were cultured for 3 days with irradiated 721.221 cells expressing HLA-A*2402 (.221-A*2402) or irradiated 721.221 expressing HLA-B*5201 (.221-B*5201) *in vitro*. As shown in Figure 5B, the human CD8$^+$ T cells and the CD8$^-$ T cells (mostly CD4$^+$ T cells) reconstituted in the mice did not proliferate in response to.221-A*2402 or. 221-B*5201 cells. In addition, they did not proliferate in response to splenocytes of C57BL/6(H-2^b) or Balb/c (H-2^d) mice (data not shown). These results together indicate that the human CD8$^+$ T cells could not recognize alloantigens in the mice.

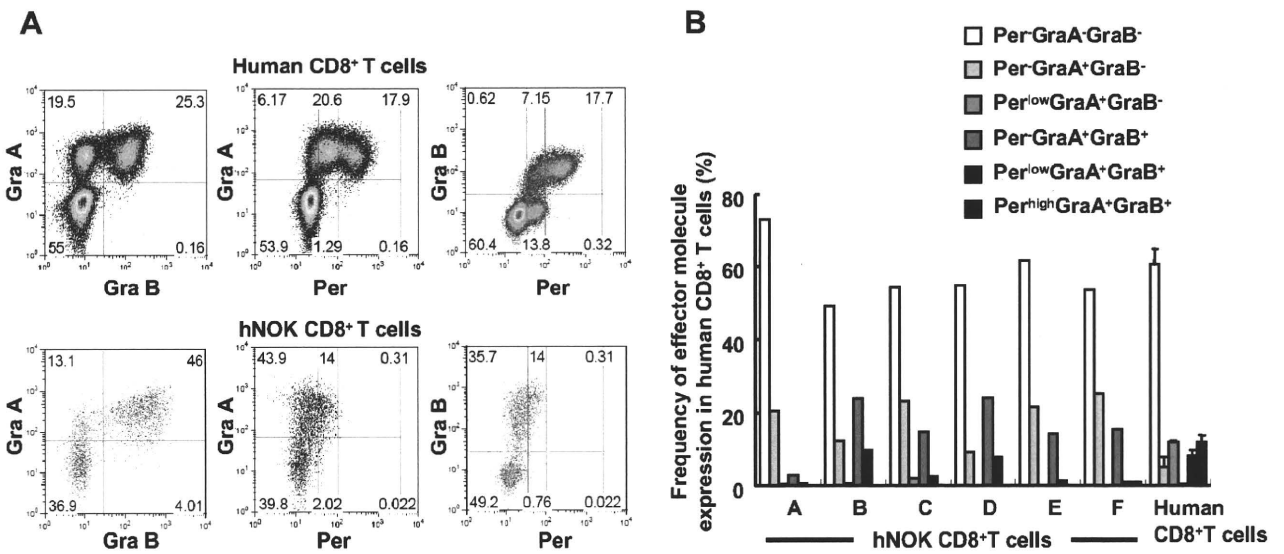

Figure 3. Expression of 3 cytolytic effector molecules of human CD8$^+$ T cells reconstituted in hNOK mice. Human CD8$^+$ T cells among splenocytes of hNOK mice were analyzed for the expression of 3 cytolytic effector molecules: Per, Gra A, and Gra B. (A) A representative result showing co-expression of 3 cytolytic effector molecules in human CD8$^+$ T cells from a hNOK mouse (lower data) and a human adult individual (upper data). (B) Summarized result for the expression of 3 the cytolytic effector molecules in human CD8$^+$ T cells from hNOK mice (n = 6) and human adult individuals (n = 3).
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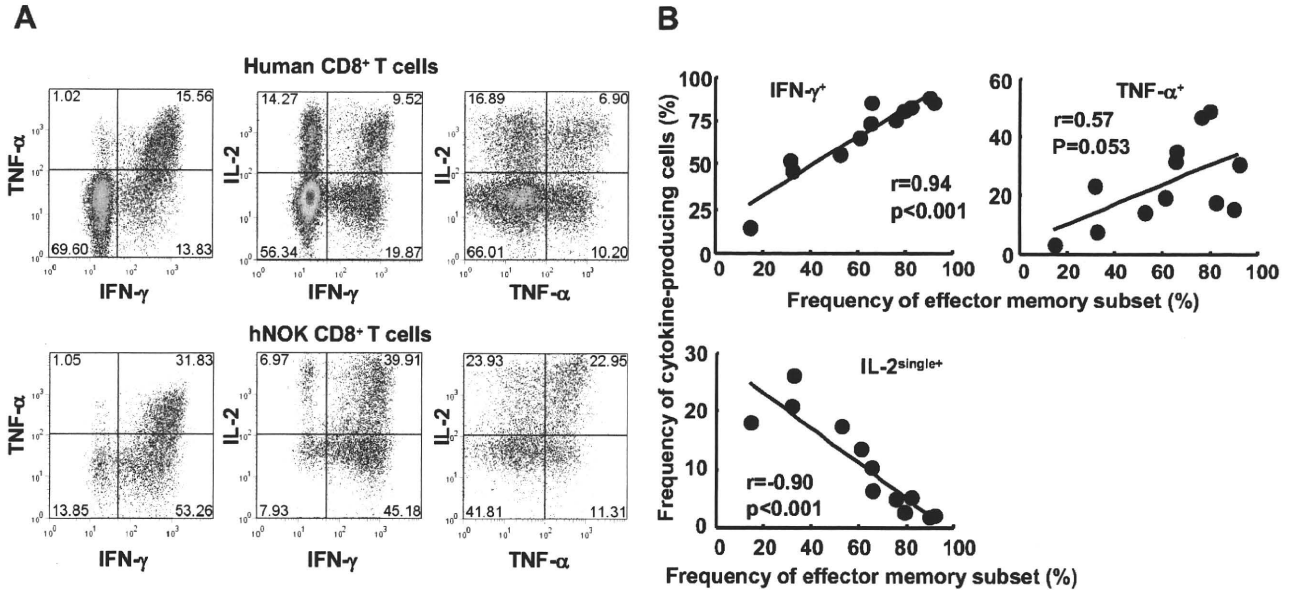

Figure 4. Cytokine production of human CD8$^+$ T cells reconstituted in hNOK mice. Splenocytes from hNOK mice were cultured with PMA and ionomycin for 6 hours and then the production of IFN-γ, TNF-α, and IL-2 cytokines by human CD8$^+$ T cells was measured by flow cytometry. (A) A representative result for cytokine production by human CD8$^+$ T cells from a hNOK mouse (lower data) and a human adult individual (upper data). (B) Correlation between frequency of IFN-γ-, TNF-α-, and IL-2$^+$IFN-γ$^-$TNF-α$^-$ (IL-2$^{single+}$)-producing CD8$^+$ T cells and that of effector memory (CD27$^+$CD28$^+$CCR7$^-$CD45RA$^-$ plus CD27^{low}CD28$^-$CD45RA$^{+/-}$CCR7$^-$) CD8$^+$ T cell subset (n = 12).
doi:10.1371/journal.pone.0013109.g004

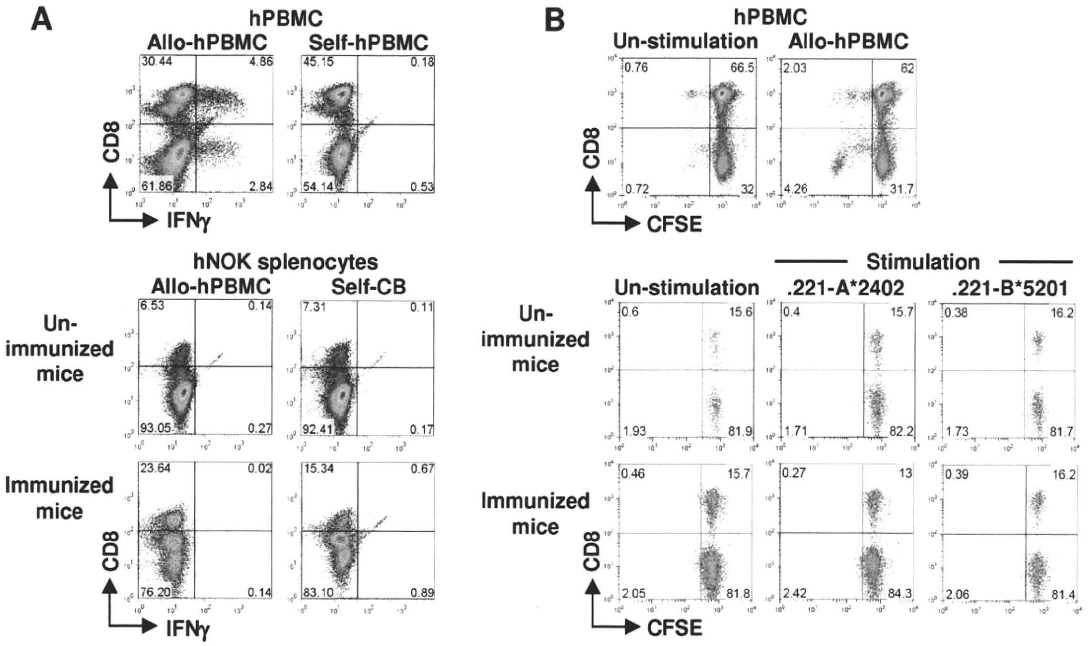

Figure 5. Alloreactivity of human CD8$^+$ T cells reconstituted in hNOK mice. The hNOK mice were immunized for 31 days with irradiated human PBMC (allo-hPBMC) from a healthy donor with HLA-A*2402/A*2402, HLA-B*5201/B*5901, and HLA-DRB1*1502/DRB1*0405 (immunized mice) or PBS (un-immunized mice). The splenocytes of each hNOK mice were harvested, and the cells were stained by using anti-human CD45, anti-human CD3, and anti-human CD8 mAbs. (A) Human adult PBMC from a healthy donor with HLA-A*2601/A*2403, HLA-B*3501/B*5101, and HLA-DRB1*0405/DRB1*0405 were cultured with the allo-hPBMC or the self-hPBMC for 7 days *in vitro*, and a representative result showing the IFN-γ production by the human CD8$^+$ T cells and the human CD8$^-$ T cells is shown as a control (upper data). The splenocytes from the immunized hNOK mice were cultured with the allo-hPBMC or self-CB for 7 days *in vitro*, and a representative result for IFN-γ production by the human CD8$^+$ T cells and the human CD8$^-$ T cells is shown (lower data). (B) Splenocytes from the immunized hNOK mice were cultured with irradiated 721.221 cells expressing HLA-A*2402 (.221-A*2402) or irradiated 721.221 expressing HLA-B*5201 (.221-B*5201) for 3 days *in vitro*, and a representative result for the proliferation of the human CD8$^+$ T cells or the human CD8$^-$ T cells is shown. One representative experiment from 3 experiments is shown.
doi:10.1371/journal.pone.0013109.g005