### *5.2. Immune-Mediated Attenuation of Nef Function?*

HIV-1 Nef is highly targeted by the host immune response during primary infection [119], and a large number of HLA-associated polymorphisms have been identified within or near known CTL epitopes [120]. HLA-mediated immune pressure on Nef drives rapid selection of escape mutations following infection [121,122], and HLA-associated polymorphisms have been identified at approximately half of Nef's 206 residues [120,123]. Indeed, a substantial proportion of natural sequence variation observed in Nef is attributable to immune selection pressure on this protein [120,124]. It has been presumed that the extensive ability of Nef to incorporate sequence changes would allow it to escape from immune pressure with limited consequence for viral fitness. However, while the impact of certain CTL escape mutations on Nef function has been assessed [35,125,126], the broader impact of HLA-restricted pressure on Nef function and viral pathogenesis at the individual or population level has not been clarified, and data to address this important issue are currently lacking.

We have previously reported that HLA-B*35-associated CTL escape mutations R75T and Y85F located in the conserved proline-rich region of Nef can impair HLA-I down-regulation activity [35,126]. Along with other data for A*02 [125], these observations indicate that host immune pressure can alter Nef function in at least some cases and further highlight the need for additional studies to characterize the wide array of Nef sequence variants that are likely to arise within an individual during natural infection. Indeed, population-level analyses have identified a number of HLA-associated polymorphisms in the N-terminal and C-terminal domains of Nef, as well as in sites near other critical Nef residues [120], including several that are selected by the protective allele HLA-B*57. Fully understanding the potential impact of naturally occurring HLA-associated mutations on Nef function and clinical outcome will be an important area for future study.

## 6. Conclusions

Research advances have significantly improved our understanding of the HIV-1 Nef protein and the mechanisms that it uses to effectively down-regulate HLA class I expression on the surface of infected cells. Molecular and biochemical studies have identified many of Nef's crucial binding partners and have mapped Nef sequence motifs that are required for its function. Structural data have very recently allowed us to visualize the Nef protein in complex with HLA-I and the μ1 subunit of AP-1, validating our current models and identifying potential sites for therapeutic intervention.

Nef-mediated evasion of host immunity is expected to contribute significantly to the establishment and maintenance of persistent HIV-1 infection. However, despite recent progress in the field to understand the cellular mechanisms of Nef-mediated HLA-I down-regulation, our knowledge of Nef's role during HIV-1 disease progression remains poor. To fully elucidate the impact of Nef during natural infection, it will be necessary to extend our current studies of lab-adapted viral isolates to include detailed analyses of patient-derived Nef proteins. Only then will we be able to fully appreciate the consequence of Nef sequence variation on protein function and make important links to clinical outcome.

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**Conflict of Interest**

The authors declare no conflict of interest.

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*Correction*

# Brockman, M.A., *et al.*, Human Leukocyte Antigen (HLA) Class I Down-Regulation by Human Immunodeficiency Virus Type 1 Negative Factor (HIV-1 Nef): What Might We Learn From Natural Sequence Variants? *Viruses* 2012, *4*, 1711-1730

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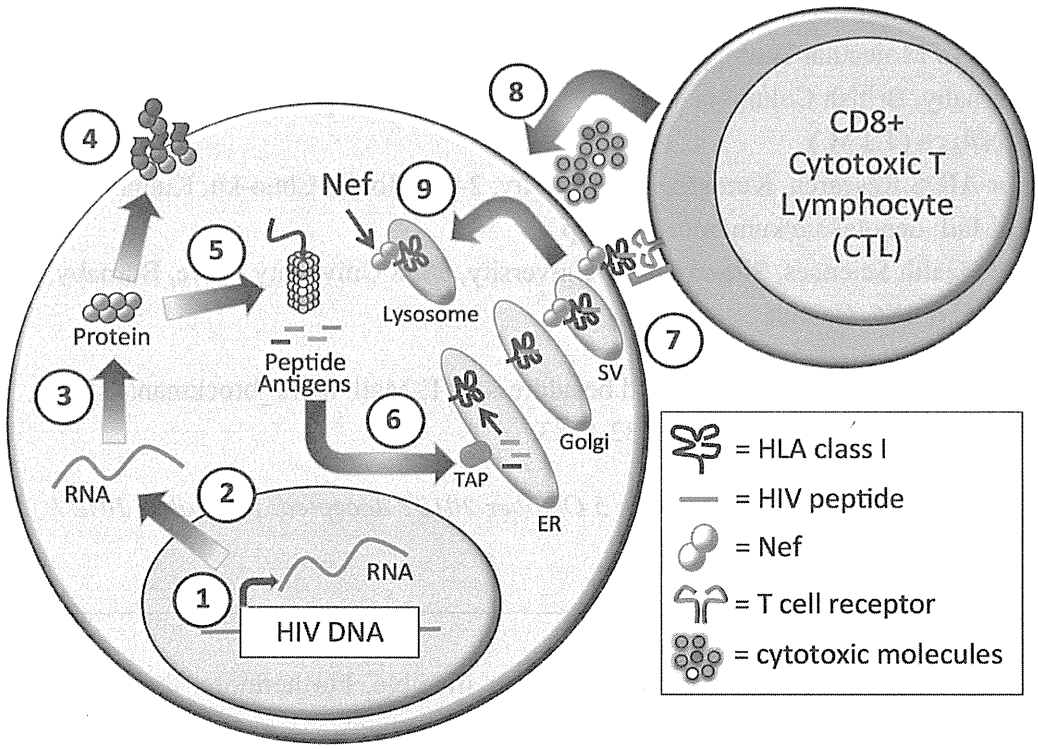
In the original manuscript, the text in figure 1 is illegible. Furthermore, there is an unnecessary carriage return (page 1716, ~line 18) "crystallographic ... methods".

"...The N-terminal anchor domain of Nef is required for membrane association and localization into detergent-insoluble "lipid rafts" [77], while the central core encodes numerous protein interaction and intracellular trafficking motifs that contribute differentially to diverse Nef functions [78]. The central core domain of Nef adopts a stable tertiary fold, permitting its early characterization using both NMR and X-ray crystallographic methods [79,80]. Our understanding of Nef-mediated HLA down-regulation has been significantly enhanced by the recently reported crystal structure of Nef protein in complex with the MHC-I cytoplasmic domain and the μ1 subunit of the clatherin AP1 complex [76]."

The correct figure should be:

**Figure 1.** Presentation of viral peptide antigens by Human Leukocyte Antigen (HLA) class I. Human immunodeficiency virus type 1 (HIV-1) proviral gene expression, including RNA transcription (**a**) and protein translation (**b**); generates functional viral proteins (**c**) as well as truncated or mis-folded proteins that are degraded by the cellular proteasome complex to form short antigenic peptides (**d**); These peptides are transported from the cytoplasm into the endoplasmic reticulum (ER) (**e**) where they can be loaded onto HLA-I molecules. Peptide/HLA complexes traffic from the ER through the Golgi and secretory vesicle (SV) network to the plasma cell membrane, where the peptide antigens are presented to circulating cytotoxic T lymphocytes (CTL) (**f**); The viral Nef protein shuttles HLA molecules located at the cell surface or within the *trans*-Golgi network into lysosomal compartments (**g**); where they are degraded. In the absence of Nef-mediated HLA down-regulation, antigen-specific CTL respond to stimulation by releasing cytotoxic molecules, including perforin and granzymes, resulting in elimination of the virus-infected cell (h).

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1. Mwimanzi, P.; Markle, T.J.; Ueno, T.; Brockman, M.A. Human Leukocyte Antigen (HLA) Class I Down-Regulation by Human Immunodeficiency Virus Type 1 Negative Factor (HIV-1 Nef): What Might We Learn From Natural Sequence Variants? *Viruses* **2012**, *4*, 1711-1730.

PLOS ONE

# Expansion of Activated Memory CD4$^+$ T Cells Affects Infectivity of CCR5-Tropic HIV-1 in Humanized NOD/SCID/JAK3$^{null}$ Mice

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## Abstract

Humanized mice reconstituted with human hematopoietic cells have been developed as an experimental animal model for human immunodeficiency virus type 1 (HIV-1) infection. Myeloablative irradiation is usually performed to augment the engraftment of donor hematopoietic stem cells (HSCs) in recipient mice; however, some mouse strains are susceptible to irradiation, making longitudinal analysis difficult. We previously attempted to construct humanized NOD/SCID/JAK3$^{null}$ (hNOJ) mice, which were not irradiated prior to human HSC transplantation. We found that, over time, many of the reconstituted CD4$^+$ T cells expanded with an activated effector memory phenotype. Therefore, the present study used hNOJ mice that were irradiated (hNOJ (IR+)) or not (hNOJ (IR−)) prior to human HSC transplantation to examine whether the development and cellularity of the reconstituted CD4$^+$ T cells were influenced by the degree of chimerism, and whether they affected HIV-1 infectivity. Indeed, hNOJ (IR+) mice showed a greater degree of chimerism than hNOJ (IR−) mice. However, the conversion of CD4$^+$ T cells to an activated effector memory phenotype, with a high percentage of cells showing Ki-67 expression, occurred in both hNOJ (IR+) and hNOJ (IR−) mice, probably as a result of lymphopenia-induced homeostatic expansion. Furthermore, when hNOJ (IR+) and hNOJ (IR−) mice, which were selected as naïve- and memory CD4$^+$ T cell subset-rich groups, respectively, were infected with CCR5-tropic HIV-1 *in vivo*, virus replication (as assessed by the plasma viral load) was delayed; however, the titer subsequently reached a 1-log higher level in memory-rich hNOJ (IR−) mice than in naïve-rich hNOJ (IR+) mice, indicating that virus infectivity in hNOJ mice was affected by the different status of the reconstituted CD4$^+$ T cells. Therefore, the hNOJ mouse model should be used selectively, i.e., according to the specific experimental objectives, to gain an appropriate understanding of HIV-1 infection/pathogenesis.

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## Introduction

Human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS) in humans, infects CD4$^+$ T cells as well as macrophages and dendritic cells by binding to its primary receptor, CD4, and a co-receptor, usually CCR5 or CXCR4 [1,2,3]. Not only is the tropism of HIV-1 determined by its use of either CCR5 or CXCR4, but factors such as cellular activation status, differentiation and maturation status, cell type, and the histological location of the target cells also determine HIV-1 infectivity with respect to its replication, dissemination, and latency [4,5,6]. *In vivo* studies are essential if we are to better understand the dynamics of HIV-1 infection and pathogenesis, in addition to improving the trials of putative anti-HIV/AIDS drugs, gene therapy, and vaccines. Therefore, the development of suitable experimental animal models is desirable. Mice reconstituted with human hematopoietic cells, referred to as humanized mice, have recently attracted attention as experimental animal models of HIV-1 infection [7,8,9,10,11,12].

At present, bone marrow/liver/thymus (BLT) mice, which are produced by surgical implantation of human fetal thymus and liver tissue into NOD/SCID mice, followed by transplantation of autologous fetal liver CD34$^+$ hematopoietic stem cells (HSCs), seem to be an ideal humanized mouse model because they support T cell development in a human thymic environment and generate human MHC-restricted T cell responses *in vivo* [13]. However, due to the ethical issues surrounding the use of fetal organs, studies using BLT mice are limited. Therefore, Rag2$^{null}$IL2R$\gamma^{null}$ mice (including BRG (BALB/c-background) and B6RG (C57BL/6-background) mice), or NOD/SCID/IL2R$\gamma^{null}$ mice (including NOG (truncated IL-2R$\gamma$ chain lacking the intracytoplasmic domain) and NSG (complete absence of IL-2R$\gamma$ chain) mice) are conventionally used as recipients of transplanted human HSCs [7,9,10,12,14,15,16]. In addition, BALB/c-Rag1$^{null}$IL2R$\gamma^{null}$ mice [17] and NOD/SCID/JAK3$^{null}$ (NOJ) mice [18] have

recently been developed as an alternative recipient mouse strain, thereby providing more options for the construction of humanized mice.

Various methods are used to construct humanized mice [12,14,19]. The key issue is the efficiency of HSC engraftment [19,20,21]. Myeloablative irradiation is conventionally performed to augment the engraftment of donor HSCs within the recipient bone marrow (BM) [22], although irradiation may shorten the life-span of certain strains of mice [17,18,22,23]. Hence, it is difficult to study prolonged HIV-1 infection/pathogenesis using certain strains of irradiated mice. To overcome this problem, Watanabe *et al.* proposed the use of non-irradiated humanized NOG mice, as they have a longer life-span and support HIV-1 infection for over 3 months [22]. For this reason, we attempted to construct a humanized mouse model based on NOJ mice (hNOJ mice) that were not irradiated prior to HSC transplantation. Our preliminary study showed that many of the $CD4^+$ T cells that were reconstituted in hNOJ mice expanded with an activated effector memory phenotype over time. Because non-irradiated humanized mice reconstitute human hematopoietic cells less efficiently [22], non-irradiated hNOJ mice may provide a lymphopenic environment that favors lymphopenia-driven homeostatic proliferation (HSP) of T cells. Lymphopenia-induced HSP involves both slowly and rapidly proliferating $CD4^+$ T cells: the former remain phenotypically naïve, whereas the latter convert from a naïve to a memory-like phenotype with a greater activation potential [24,25,26]. The occurrence of T cell HSP, particularly in the latter case, is supported by other conventional humanized mouse models based on the BRG [27] and NOG [28] strains. Therefore, it is postulated that both the manner and dynamics of HIV-1 infection in humanized mice may be affected by the presence of HSP and, if so, that the humanized mouse model should be used selectively according to the specific experimental objectives.

The aim of the present study was to elucidate whether HSP of $CD4^+$ T cells was influenced by the degree of chimerism, and whether it affected HIV-1 infectivity in hNOJ mice. First, we compared the dynamics of reconstituted $CD4^+$ T cell cellularity between hNOJ mice that were irradiated (hNOJ (IR+)) or not (hNOJ (IR−)) prior to human HSC transplantation, and characterized them as high and low chimerism groups, respectively. Here, we show that the conversion of $CD4^+$ T cells to an activated effector memory phenotype occurred in both hNOJ (IR+) and hNOJ (IR−) mice over time. We also challenged hNOJ (IR+) and hNOJ (IR−) mice, which were selected as naïve- and memory $CD4^+$ T cell subset-rich groups, respectively, with CCR5-tropic (R5) HIV-1. The plasma viral load was blunted during the early phase post-challenge, but subsequently reached a 1-log higher level in memory-rich hNOJ (IR−) mice than in naïve-rich hNOJ (IR+) mice. Taken together, the results of the present study provide useful information for evaluating the usefulness of hNOJ mice as a model of HIV-1 infection.

## Methods

### Ethics Statement

Human umbilical cord blood was obtained from the Tokyo Cord Blood Bank (Tokyo, Japan) after receiving written informed consent. Human peripheral blood was obtained from the Blood Bank of Japan Red Cross (Tokyo, Japan) or from healthy adult volunteers after receiving written informed consent. The use of human umbilical cord blood and peripheral blood was approved by the Institutional Ethical Committee of the National Institute of Infectious Diseases (Tokyo, Japan) (Permit Numbers: 127 and 122, respectively). All mice were treated in accordance with the guidelines set down by and approved by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (Permit Numbers: 208022, 109019, 110026, 211033, and 112040).

### Mice

NOD/SCID/JAK3$^{null}$ (NOJ) mice were established as described previously [18] and maintained under specific pathogen-free conditions in the animal facility at the National Institute of Infectious Diseases.

### Construction of Humanized Mice

Human HSCs were isolated from umbilical cord blood using a CD133 MicroBead Kit (Miltenyi Biotec Inc, Auburn, CA). The purity was approximately 90% as assessed by flow cytometric analysis of CD34 expression. Human HSCs ($0.5-1\times10^5$ cells) were transplanted into the livers of irradiated (1 Gy) or non-irradiated newborn mice within 2 days of birth. The number of hNOJ mice used and the ID number of the donor from which hNOJ mice were derived are listed in Table 1.

### Cell Preparation

Human peripheral blood mononuclear cells (PBMCs) were separated by a Ficoll-Hypaque density gradient (Lymphosepal; IBL, Gunma, Japan). For hNOJ mice, peripheral blood, spleens and BM were collected and the red blood cells were lysed in ACK buffer (0.15 M $NH_4Cl$, 1 mM $KHCO_3$, and 0.1 mM EDTA-2Na; pH 7.2−7.4). In some cases, $CD4^+$ T cells from human PBMCs or hNOJ splenocytes were negatively selected using an EasySep Human $CD4^+$ T cell Enrichment Kit (StemCell Technologies, Vancouver, BC, Canada), or a combination of this kit and a StemSep Mouse/Human Chimera Enrichment Kit (StemCell Technologies), respectively. The purity was ≥95% as assessed by flow cytometry.

### Flow Cytometry

Cells were stained with fluorescence-conjugated monoclonal antibodies as described previously [29]. The following antibodies were used for flow cytometry in various combinations: FITC-conjugated anti-mouse CD45 (30-F11), anti-human CD34 (581), and CD195/CCR5 (HEK/1/85a); PE-conjugated anti-human CD19 (HIB19), CD150 (A12(7D4)), CD184/CXCR4 (12G5) and IFN-γ (4S.B3); PerCP-conjugated anti-human CD3 (UCHT1), CD4 (RPA-T4), CD8a (RPA-T8), and HLA-DR (L243); PE-Cy7-conjugated anti-human CD3 (UCHT1); APC-conjugated anti-human CD8a (RPA-T8) and CD45RA (HI100); Alexa Fluor 647-conjugated anti-human CD25 (BC96); Alexa Fluor 700-conjugated anti-human CD4 (OKT4), CD27 (O323) and CD69 (FN50); Pacific Blue-conjugated anti-human CD3 (UCHT1), CD4 (RPA-T4), and CD45 (HI30) (all purchased from BioLegend, San Diego, CA). FITC-conjugated anti-human Ki-67 (B56) and PE-Cy7-conjugated anti-human CD197/CCR7 (3D12) were purchased from BD Biosciences (San Diego, CA). APC-conjugated anti-human CD14 (TÜK4) was purchased from Miltenyi Biotec Inc. Anti-human CD11a (TS1/22.1.1.13) and CD38 (OKT10) antibodies were prepared from hybridoma cells (ATCC Nos. HB202 and CRL8022, respectively) and were conjugated with Alexa Fluor 647 and Alexa Fluor 700, respectively, using Alexa Fluor succinimidyl esters (Invitrogen, Carlsbad, CA). Dead cells were stained with propidium iodide or with a LIVE/DEAD Fixable Dead Cell Stain Kit (L34957; Invitrogen) and were gated out during analysis. Intracellular staining for Ki-67 and IFN-γ was performed using a BD Cytofix/Cytoperm Fixation/Permeabiliza-

**Table 1.** The number of mice used in the present study.

| Figure | Number of NOJ mice | | | | Notes |
|---|---|---|---|---|---|
| | IR+ | | IR− | | |
| | Total | Composition (donor #) | Total | Composition (donor #) | |
| Figure 1 | | | | | |
| A | 16 | 2 (D56), 3 (D57), 3 (D59), 1 (D62), 1 (D63), 1 (D65), 1 (D69), 1 (D74), 3 (D80) | 28 | 3 (D47), 2 (D49), 2 (D51), 1 (D52), 2 (D56), 2 (D57), 3 (D59), 2 (D65), 3 (D67), 1 (D68), 2 (D69), 1 (D74), 4 (D80) | |
| B | 7 | 3 (G65), 1 (G69), 3 (G80) | 10 | 3 (D65), 3 (D69), 4 (D80) | |
| Figure 2 | | | | | |
| A, D, E | 22 | 1 (D56), 3 (D57), 3 (D59), 4 (D62), 5 (D63), 3 (D65), 3 (D80) | 13 | 2 (D56), 2 (D57), 2 (D59), 3 (D65), 4 (D80) | |
| B, C | 5 | 1 (D54), 2 (D60), 1 (D104), 1 (D105) | 6 | 4 (D54), 1 (D60), 1 (D110) | |
| Figure 3 | | | | | |
| A | 1[a] | 1 (D65) | | | [a]Representative of Figure 3B |
| B | 18 | 3 (D57), 2 (D59), 2 (D62), 5 (D63), 3 (D65), 1 (D69), 2 (D80) | 6 | 3 (D47), 2 (D57), 1 (D80) | |
| Figure 4 | | | | | |
| A | 18 | [b] | 6 | [b] | [b]Same as in Figure 3B |
| B | 2 | 2 (D114) | 4 | 1 (D1), 2 (D3), 1 (D4) | |
| C | 1[c] | 1 (D80) | | | [c]Representative of 4 IR+ mice |
| D | 2 | 2 (D80) | 2 | 2 (D80) | |
| E | 25 | 3 (D57), 3 (D59), 4 (D62), 5 (D63), 2 (D64), 3 (D65), 2 (D69), 3 (D80) | 21 | 3 (D47), 2 (D57), 3 (D59), 3 (D65), 3 (D67), 1 (D68), 3 (D69), 3 (D80) | |
| Figure 5 | | | | | |
| A | 12 | 3 (D65), 1 (D90), 6 (D101), 1 (D113), 1 (D114) | 8 | 2 (D59), 1 (D65), 1 (D74), 1 (D85), 3 (D92) | |
| B | 6 | 2 (D80), 1 (D101), 2 (D113), 1 (D114) | 6 | 3 (D80), 3 (D92) | |
| C | 1[d] | 1 (G113) | | | [d]Representative of Figure 5D |
| D | 3 | 2 (D113), 1 (D114) | 3 | 3 (D92) | |
| Figure 6 | | | | | |
| A, B | 18 | [b] | 6 | 2 (D57), 1 (D59), 2 (D69), 1 (D80) | [b]Same as in Figure 3B |
| C | 1[e] | 1 (D65) | | | [e]Representative of Figure 6D |
| D, E | 3 | 3 (D65) | 1 | 1 (D59) | |
| Figure 7 | | | | | |
| A, B, C | 7 | 7 (D101) | 8 | 2 (D13), 1 (D23), 5 (D122) | |
| D | 6[f] | 6 (D101) | 7[f] | 2 (D13), 1 (D23), 4 (D122) | [f]Included in Figure 7A, B, C |
| Figure 8 | | | 3 | 3 (D33) | |

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tion Solution (BD Biosciences) or a FIX & PERM Fixation and Permeabilization Kit (Invitrogen). Absolute cell counts in the peripheral blood of hNOJ mice were determined using Trucount tubes (BD Biosciences). Data were collected using a FACSCanto II (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences) or FlowJo software (Tree Star, San Carlos, CA).

## *Ex vivo* IFN-γ Production in $CD4^+$ T Cells Induced by PMA/ionomycin Stimulation

Purified $CD4^+$ T cells were stimulated *ex vivo* with or without 20 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, Mo) and 1 μg/ml ionomycin (Sigma-Aldrich) in RPMI medium containing 10% heat-inactivated fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 5 μg/ml brefeldin A, and 2 μM monensin at 37°C for 4 h. Intracellular IFN-γ was analyzed by flow cytometry as described above. Because PMA treatment downmodulates CD4 expression [30], and to distinguish $CD4^+$ T cells from $CD8^+$ T cells (a minor contaminant in the purified $CD4^+$ T cell fraction), $CD3^+CD8^-$ T cells were denoted as $CD4^+$ T cells in this experiment.

## Detection of Cytokines in the Plasma

IL-2, IL-7, and IL-15 levels in the plasma from routinely collected peripheral blood samples were measured using a Milliplex MAP Human Cytokine/Chemokine Panel (Merck Millipore Japan, Tokyo, Japan) on a MAGPIX platform (Merck Millipore Japan).

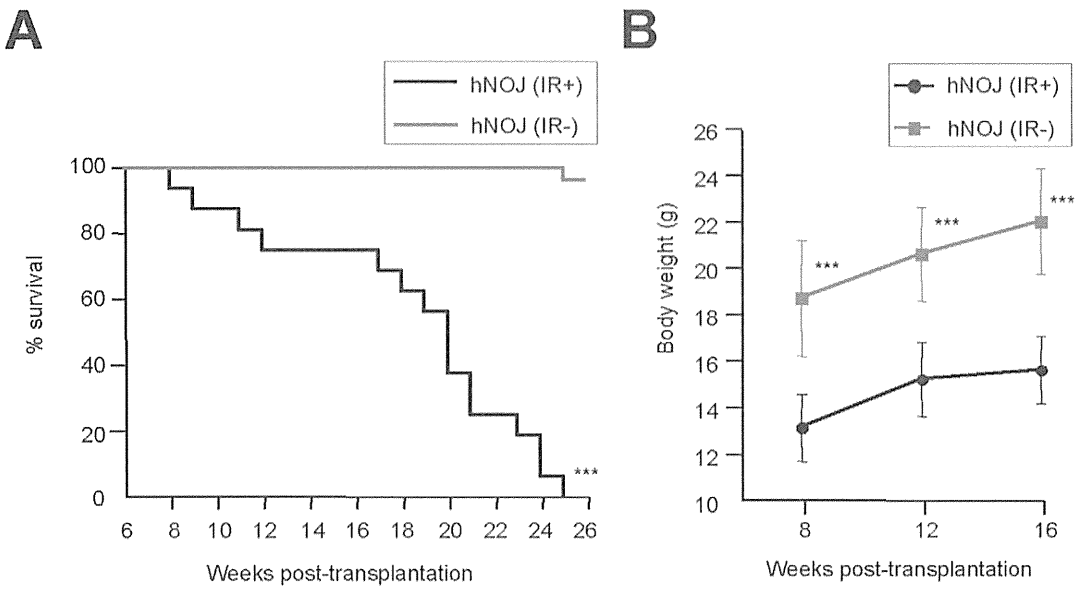


**Figure 1. Influence of irradiation on the survival and growth of hNOJ mice.** Newborn NOJ mice (1−2 days after birth) were irradiated (1 Gy) or not before transplantation with $CD34^+CD133^+$ HSCs isolated from human cord blood. (A) Survival curves for hNOJ (IR+) and hNOJ (IR−) mice ($n=16$ and $n=28$, respectively). Significant differences ($^{***}P<0.001$) were determined by the log-rank test. (B) Changes in the body weight of hNOJ (IR+) and hNOJ (IR−) mice ($n=7$ and $n=10$, respectively). Data are expressed as the mean ± SD. Significant differences ($^{***}P<0.001$) were determined by the unpaired $t$ test.
doi:10.1371/journal.pone.0053495.g001

### Fusion Assay

A fusion assay was performed using HIV-1 possessing β-lactamase-Vpr chimeric proteins (BlaM-Vpr) and $CD4^+$ T cells loaded with CCF2 dye, a fluorescent substrate for β-lactamase, as previously described [31,32]. In brief, R5 HIV-$1_{NL\text{-}AD8\text{-}D}$ [29] containing BlaM-Vpr (HIV-$1_{NL\text{-}AD8\text{-}D\text{-}BlaM\text{-}Vpr}$) was obtained by cotransfecting 293T cells with pNL-AD8-D plus pMM310, encoding *Escherichia coli* β-lactamase fused to the amino terminus of Vpr [33]. The purified $CD4^+$ T cells ($1\times10^6$ cells) were infected with 200 ng of p24-measured amounts of HIV-$1_{NL\text{-}AD8\text{-}D\text{-}BlaM\text{-}Vpr}$ by spinoculation at $1200\times g$ for 2 h at 25°C as previously described [34]. After spinoculation, cells were washed and then incubated in RPMI containing 10% heat-inactivated fetal bovine serum for 2 h at 37°C to induce viral fusion. After fusion, cells were washed and loaded with CCF2-AM for 1 h at RT using a GeneBLAzer *In Vivo* Detection Kit (Invitrogen). The dye-loaded cells were incubated overnight at RT and subjected to flow cytometry. Cells permissive for HIV-1 fusion were detected at a fluorescence emission spectrum of 447 nm after excitation with a 405-nm violet laser in a FACSCanto II.

### *In vivo* HIV-1 Infection of hNOJ Mice

hNOJ mice were challenged intravenously with 200 ng of p24-measured amounts of R5 HIV-$1_{NL\text{-}AD8\text{-}D}$, which express DsRed [29]. Peripheral blood was harvested from the HIV-1-challenged hNOJ mice on a weekly basis. All animal experiments with highly pathogenic viruses were conducted in a biosafety level 3 containment facility.

### Detection of Plasma Viral RNA by Quantitative Real-time RT-PCR

Viral RNA was extracted from the plasma and purified using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). The RNA was subjected to quantitative real-time RT-PCR using a SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen) with the following set of HIV-1 gag primers and probe [35]: forward primer, HIVgag638 (+) (5′-CTCTCGACGCAGGACTCGGCTTGCT-3′); reverse primer, HIVgag803 (−) (5′-GCTCTCGCACCCATCTCTCTCCTTC-TAGCC-3′); and HIV-1 gag probe, TaqMan 720R748 (FAM-5′-GCAAGAGGCGAGRGGCGGCGACTGGTGAG-3′-BHQ-1). PCR was performed using an Mx3000P PCR system (Stratagene, La Jolla, CA). The detection limit was set at 5000 copies/ml plasma using samples obtained from HIV-$1_{NL\text{-}AD8\text{-}D}$-challenged NOJ mice that were not transplanted with HSCs.

### Statistical Analysis

The significance of the data was evaluated using an unpaired $t$ test, a paired $t$ test, the Mann-Whitney U test, the Wilcoxon signed rank test, Spearman's rank correlation coefficient, or Tukey's or Bonferroni multiple comparison tests based on the normality and variance of the data compared, or the Log-rank test (see individual Figure Legends). Prism ver.4 software (GraphPad Software, Inc., San Diego, CA) was used for all analyses. $P<0.05$ was considered statistically significant.

## Results

### Influence of Irradiation on the Survival and Growth of hNOJ Mice

We initially examined how the irradiation of recipient mice influences their survival and growth. Because infant mice were sometimes cannibalized and abandoned by their mothers, and the death of an infant could not always be attributed to irradiation, we started monitoring weaned mice from 6 wk post-transplantation. There was a significant difference between the survival curves of irradiated hNOJ (hNOJ (IR+)) and non-irradiated hNOJ (hNOJ (IR−)) mice ($n=16$ and $n=28$, respectively, $P<0.001$) (Figure 1A). At 16 wk post-transplantation, the survival rate of hNOJ (IR+)

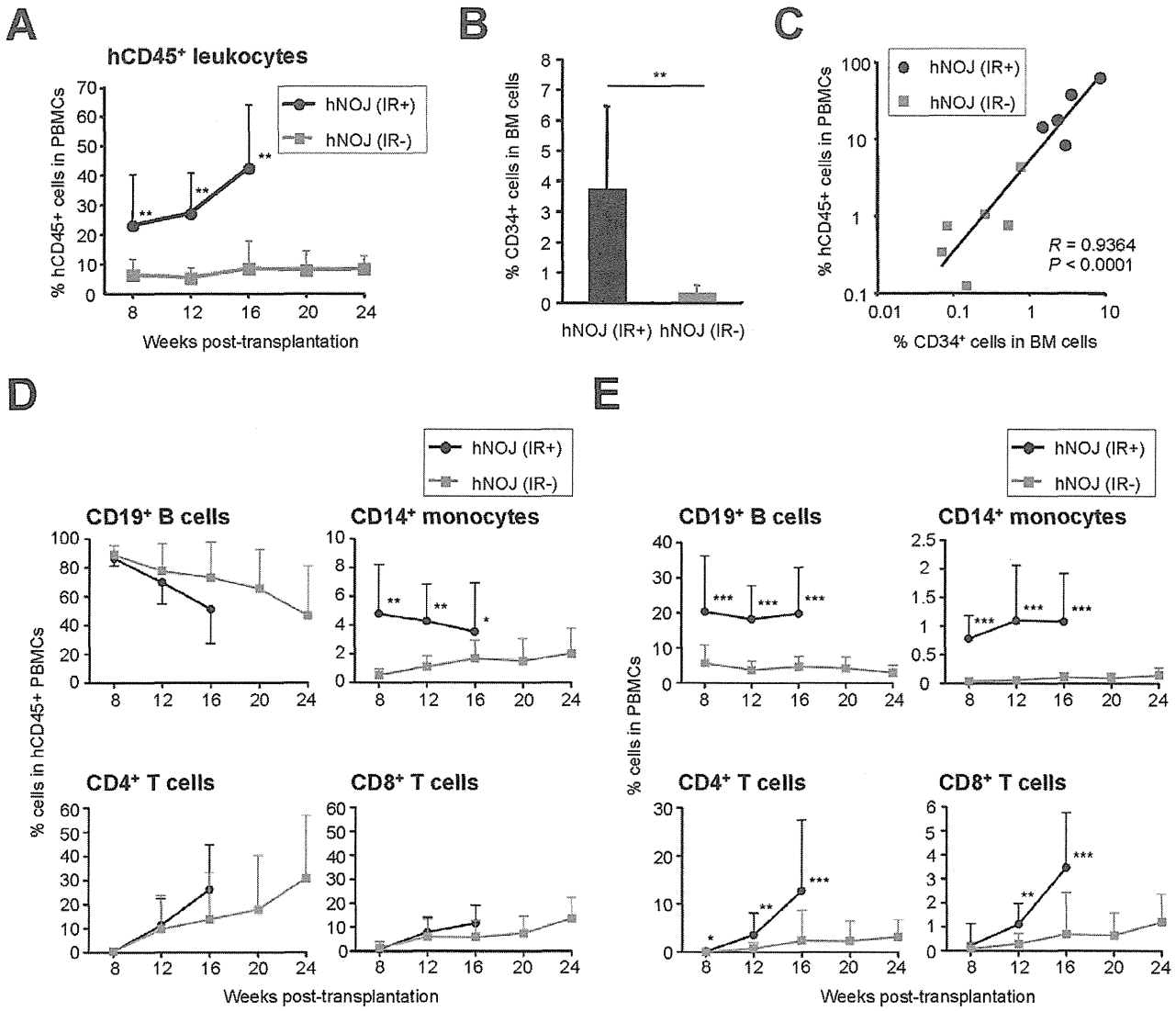


**Figure 2. Development of human hematopoietic cells in hNOJ mice.** (A) Changes in the percentage of human CD45$^+$ (hCD45$^+$) cells within the PBMC population from hNOJ (IR+) and hNOJ (IR−) mice ($n=22$ and $n=13$, respectively). Data are expressed as the mean ± SD. Significant differences ($^{**}P<0.01$) were determined by the Mann-Whitney U test. (B) Percentage of human CD34$^+$ cells within the BM cells isolated from hNOJ (IR+) and hNOJ (IR−) mice ($n=5$ and 6, respectively) at 8 wk post-transplantation. Data are expressed as the mean ± SD. Significant differences ($^{**}P<0.01$) were determined by the Mann-Whitney U test. (C) Association between the percentage of hCD45$^+$ cells within the PBMC population and that of CD34$^+$ cells within the BM cells of hNOJ (IR+) and hNOJ (IR−) mice at 8 wk post-transplantation (11 plots from five hNOJ (IR+) and six hNOJ (IR−) mice). Spearman's rank correlation coefficient was used for statistical analysis. (D, E) Changes in the percentage of human CD19$^+$ B cells, CD14$^+$ monocytes, CD4$^+$ T cells (CD3$^+$CD4$^+$CD8$^-$ cells), and CD8$^+$ T cells (CD3$^+$CD4$^-$CD8$^+$ cells) within the peripheral blood hCD45$^+$ cell population (D) or total PBMC populstion (E) from hNOJ (IR+) and hNOJ (IR−) mice ($n=22$ and $n=13$, respectively). Data are expressed as the mean ± SD. Significant differences ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$) were determined by the Mann-Whitney U test.
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mice dramatically declined (median survival: 20.0 wk) and none survived beyond 25 wk post-transplantation (Figure 1A). However, obvious signs and symptoms of illness, such as wasting, weakness, diarrhea, hunchback posture, and alopecia, were not observed during their lifetime; although the growth of hNOJ (IR+) mice ($n=7$) was significantly stunted compared with that of hNOJ (IR−) mice ($n=10$) (Figure 1B). Although the life-span of the hNOJ (IR+) mice used in the present study was shorter than that reported previously [18], probably because of the environmental conditions in our animal facility, these results demonstrated that irradiation apparently induces high mortality and low growth in hNOJ mice. Therefore, in the present study, the averaged data obtained from hNOJ (IR+) mice surviving up until 16 wk post-transplantation are reported for all the following experiments.

## Development of Human Hematopoietic Cells in hNOJ Mice

Reconstitution of human hematopoietic cells (i.e., chimerism) in hNOJ mice was investigated by flow cytometry using peripheral blood samples collected routinely (every 4 wk) after 8 wk post-transplantation. hNOJ (IR+) mice ($n=22$) showed higher chimerism (according to the percentage of human CD45$^+$ (hCD45$^+$)

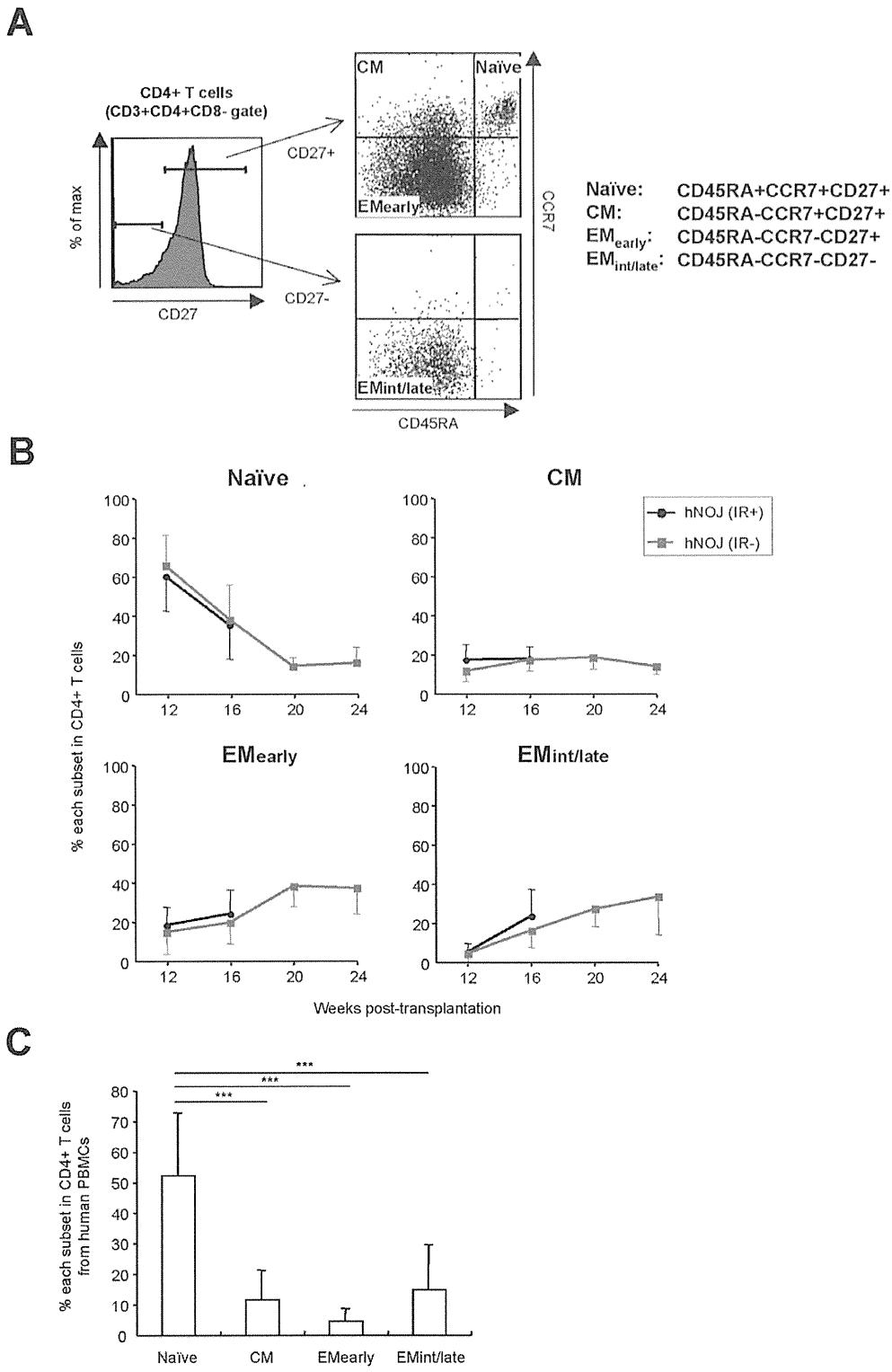


**Figure 3. Differentiation of CD4$^+$ T Cells in hNOJ mice.** (A) Naïve, CM, $EM_{early}$, and $EM_{int/late}$ subsets of CD4$^+$ T cells (gated on CD3$^+$CD4$^+$CD8$^-$) were defined as CD45RA$^+$CCR7$^+$CD27$^+$, CD45RA$^-$CCR7$^+$CD27$^+$, CD45RA$^-$CCR7$^-$CD27$^+$, and CD45RA$^-$CCR7$^-$CD27$^-$, respectively, by flow cytometry. (B) Changes in the percentage of naïve, CM, $EM_{early}$, and $EM_{int/late}$ subsets within the peripheral blood CD4$^+$ T cell populations isolated from hNOJ (IR+) and hNOJ (IR−) mice ($n = 18$ and $n = 6$, respectively). Data are expressed as the mean ± SD. (C) Percentage of CM, $EM_{early}$, and $EM_{int/late}$ subsets within human peripheral blood CD4$^+$ T cells. Data are expressed as the mean ± SD ($n = 10$). Significant differences ($^{***}P<0.001$) were determined by Tukey's multiple comparison test.
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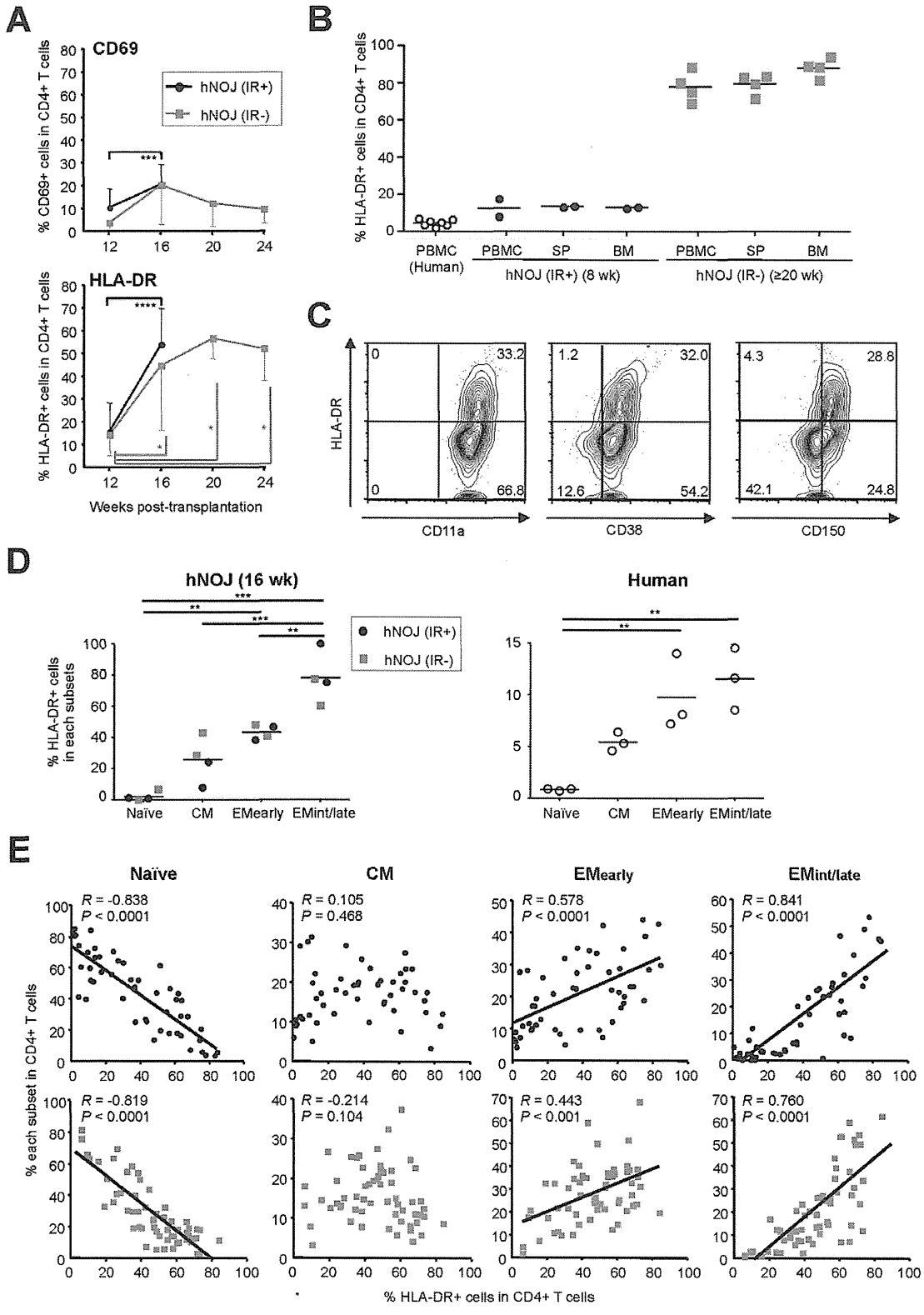


**Figure 4. Activation status of CD4$^+$ T Cells in hNOJ mice.** (A) Changes in the percentage of CD69$^+$ (upper) and HLA-DR$^+$ (lower) cells within the peripheral blood CD4$^+$ T cells isolated from hNOJ (IR+) and hNOJ (IR−) mice ($n = 18$ and $n = 6$, respectively). Data are expressed as the mean ± SD. Significant differences ($^*P<0.05$, $^{****}P<0.0001$) were determined using the Wilcoxon signed rank test or a paired $t$ test. (B) Percentage of HLA-DR$^+$ cells within the CD4$^+$ T cell populations isolated from PBMCs, spleens (SP), and BM from hNOJ (IR+) mice at 8 wk post-transplantation ($n = 2$) and hNOJ (IR−) mice at ≥20 wk post-transplantation ($n = 4$), and from human PBMCs ($n = 7$). Data are plotted individually. The black line represents the mean.