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Efficient Xenoengraftment in Severe Immunodeficient NOD/Shi-scid IL2rgnull Mice Is Attributed to a Lack of CD11c+B220+CD122+ Cells

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Xenograft animal models using immunodeficient mice have been widely applied in medical research on various human diseases. NOD/Shi-scid-IL2rgnull (NOG) mice are known to show an extremely high engraftment rate of xenotransplants compared with conventional immunodeficient mice. This high engraftment rate of xenotransplants in NOG mice was substantially suppressed by the transfer of spleen cells from NOD-scid mice that were devoid of NK cells. These results indicate that cell types other than splenic NK cells present in NOD-scid mice but not in NOG mice may be involved in this suppression. To identify the cell types responsible for this effect, we transferred subpopulations of spleen cells from NOD-scid mice into NOG mice and assessed the levels of human cell engraftment after human PBMC (hPBMC) transplantation. These experiments revealed that CD11c+B220+ plasmacytoid dendritic cells (pDCs) from NOD-scid mice markedly inhibited engraftment of human cells. The CD11c+B220+CD122+ cells further fractionated from the pDC fraction were based on the expression of CD122, which is an NK cell marker strongly inhibited during hPBMC engrafment in NOG mice. Moreover, the CD122+ cells in the pDC fraction were morphologically distinguishable from conventional CD122- NK cells and showed a higher rejection efficiency. The current results suggest that CD11c+B220+CD122+ cells play an important role in xenograft rejection, and their absence in NOG mice may be critical in supporting the successful engraftment of xenotransplants. The Journal of Immunology, 2012, 189: 4313–4320.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; CIEA, Central Institute for Experimental Animals; DC, dendritic cell; hPBMC, human PBMC; HSC, hematopoietic stem cell; IKDC, IFN-producing killer dendritic cell; KO, knockout; mDC, myeloid dendritic cell; MNC, mononuclear cell; NOG, NOD/Shi-scid-IL2rgnull; PB, peripheral blood; pDC, plasmacytoid dendritic cell; PI, propidium iodide; SIRP-α, signal regulatory protein-α.

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DCs contribute to innate and adaptive immunity and act as professional APCs that are capable of Ag uptake, processing, and presentation to naïve T cells (18, 19). DCs are classified into several populations based on surface markers and functional properties (20). Plasmacytoid dendritic cells (pDCs), characterized by the expression of CD11c and B220, represent a rare population of DCs that exists mainly in lymphoid tissues and plays a crucial role in producing type I IFNs against viruses via TLRs (21, 22). In transplant studies, prominent roles of DCs in graft rejection have been demonstrated using DC-depleted hosts (23). Thus, DCs may also suppress the reconstitution of donor cells and contribute to xenograft rejection.

In the current study, we investigated the role of DC subsets in hPBMC xenograft rejection using NOG mice. We performed transfer experiments with DC subpopulations and NK cells and demonstrated that CD11c+B220+CD122+ cells, but not other DC subpopulations and NK cells obtained from NOD-scid mice, are potent inhibitors of hPBMC engraftment in NOG mice.

Materials and Methods

Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Central Institute for Experimental Animals (CIEA) (certification number 11004A, February 16, 2011) and were performed in accordance with guidelines set forth by CIEA.

All experiments using human resources were approved by the Institutional Ethical Committee of the CIEA (certification number 08-11, September 4, 2008) and performed in accordance with CIEA guidelines. Written informed consent was obtained from all subjects in the current study.

Mice

NOD/Shi-scid-IL2rnull (NOD; formal name, NOD.Cg-prkdcscidIl2rgtm1Sugi/Jc) mice were bred and maintained under specific pathogen-free conditions at the CIEA. NOD CB17-prkdcscidShi/Jic (NOD-scid) mice were purchased from Clea Japan (Tokyo, Japan). NOD-scid EGFP transgenic mice were established by backcross mating of NOD-EGFP transgenic mice (24) to NOD-scid mice. IFN-γ knockout (KO) mice were kindly provided by Dr. Y. Ikawara (The University of Tokyo, Tokyo, Japan) and backcrossed with NOD-scid mice to establish the NOD-scid IFN-γ KO mice. These mice were housed in sterilized cages and fed sterilized food and water ad libitum. These four strains of immunodeficient mice were used at the age of 8–12 wk.

Transplantation of hPBMCs

Human peripheral blood (PB) samples were obtained from healthy volunteers after acquiring their informed consent. hPBMCs were isolated by Ficoll-Hypaque (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.) density centrifugation and washed with PBS. Cells were resuspended in PBS and transplanted via the tail vein into NOG mice.

Isolation and transplantation of DC subpopulations and NK cells

The method used for isolating DC subpopulations has been described previously (25). Briefly, spleens from NOD-scid, NOD, or NOD-scid IFN-γ KO mice were minced and digested with 0.1% collagenase (Roche Diagnostics, Laval, QC, Canada) and DNase (1 mg/ml; Sigma-Aldrich, St. Louis, MO) at 37°C for 30 min. After washing with 2% FCS in PBS, the RBCs were lysed in Pharm Lyse buffer (BD Biosciences, San Jose, CA), and cells were stained with biotinylated mouse B220 Ab (BioLegend, San Diego, CA). To isolate DC subpopulations, cells were incubated with anti-biotin magnetic beads (Miltenyi Biotec, Sunnyvale, CA), and the B220+ and B220− fractions were separated on a MACS column (Miltenyi Biotec). For myeloid dendritic cell (mDC) and pDC fractionation, the B220+ cells were further reacted with anti-mouse CD11c magnetic beads (Miltenyi Biotec) and CD11c+ cells were separated on a MACS column. The enriched B220+ cells were almost all CD11c+, as these mice lack B cells. For pDCs, and CD11c+B220+CD122+ cell purification, the enriched B220+ CD122+ cells were stained with PE-labeled mouse CD11c Ab (BioLegend), PE-Cy7–labeled streptavidin (BioLegend), and FITC-labeled mouse CD122 Ab (BD Biosciences). The CD11c+B220+CD122− pDCs were sorted using the MoFlo cell sorter (Beckman Coulter, Stanford, CA), and the results were analyzed using FlowJo software (Tomy Digital Biology, Tokyo, Japan). The purity levels of the isolated mDC and pDC fractions ranged from 91 to 95% (MACS sorting), and those of the isolated pDCs and CD11c+B220+CD122+ cells ranged from 97 to 99% (MoFlo sorting). NK cells, which were designated as CD11c+B220+CD122− cells, were isolated from the B220+ cell fraction at 97% purity using the MoFlo cell sorter. The purified mDCs, pDCs, CD11c+B220+CD122− cells, and NK cells were resuspended in PBS, and 1 × 105 to 2 × 106 cells were transplanted intravenously into NOG mice 1 d before hPBMC transplantation.

Flow cytometry

Bone marrow (BM), PB, and spleen samples were obtained from mice 2–7 wk after transplantation with hPBMCs. RBCs were lysed using the Pharm Lyse buffer (BD Biosciences), and mononuclear cells (MNCs) were prepared as single-cell suspensions. MNCs were incubated for 30 min at 4°C in the dark with the appropriate Abs. The following Abs were used: allophycocyanin–Cy7–labeled human CD45, PE- and allophycocyanin-labeled mouse CD11c, PE–Cy7–labeled mouse B220, PE-labeled mouse Siglec-H, PDCA-1, and Ly49D, and allophycocyanin-labeled mouse DX5 (BioLegend). After washing with 2% FCS in PBS, the MNCs were suspended in propidium iodide (PI) solution (BD Biosciences), followed by multicolor flow cytometry (FACScanto; BD Biosciences) and analysis of the results with FACSDiva software (BD Biosciences). The purity of human leukocyte engraftment is expressed as the percentage of human CD45+ (hCD45+) cells in the total MNC population.

Cytotoxicity measurements

Cytotoxicity activity was examined using the [51Cr] release cytotoxicity assay with Yac-1 target cells (kindly provided by Dr. K. Takeda, Juntendo University, Tokyo, Japan), according to the methods described by Shultz et al. (14). Briefly, NOD-scid, nontransplanted NOG mice, and NOG mice transplanted with pDCs or mDCs from NOD-scid mice were i.p. inoculated with polyinosinic-polycytidylic acid (Sigma-Aldrich) 48 h before assaying. Splenic MNCs from these mice were cocultured with 51Cr-labeled Yac-1 target cells for 4 h at 37°C in a 5% CO2 incubator with various E/T cell ratios. Each sample was prepared in triplicate, and the culture supernatants harvested from each well were assayed in a γ-counter (ARC300; Aloka, Tokyo, Japan). The percentage specific [51Cr] release was calculated using the formula: percentage specific release = ([cpm T − cpm S] × 100), where X is the mean experimental release of [51Cr] measured in triplicate wells. Total release (T) was determined from wells with 51Cr-labeled Yac-1 cells and 1 N HCl, and spontaneous release (S) was determined from wells with 51Cr-labeled Yac-1 cells and medium.

Induction of IFN-γ from DCs in vitro

In vitro IFN-γ induction was determined according to the methods described by Vremec et al. (25). Briefly, genetically sorted pDC and mDC fractions were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) that contained 10% FCS in 96-well flat-bottom plates at 37°C in 5% CO2. To induce IFN-γ, cells were stimulated with 5 ng/ml IL-12p70 (R&D Systems, Minneapolis, MN) and 20 ng/ml IL-18 (R&D Systems) or with 10 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) for 48 h. Culture supernatants were collected and stored at −80°C until use. IFN-γ was assayed using the Mouse IFNγ Quantikine ELISA Kit (R&D Systems).

Morphological analysis

pDCs, CD11c+B220+CD122− cells, and NK cells were isolated as described earlier. For May–Gru¨ nwald Giemsa staining, the enriched subpopulations were precipitated onto silane-coated glass slides (Muto Pure Chemicals, Tokyo, Japan) by cytoospinning and were then air dried for 3 min. The slides were soaked in May–Gru¨ nwald solution (Muto Pure Chemicals, Tokyo, Japan) and 1 N HCl, and spontaneous release (S) was determined from wells with 51Cr-labeled Yac-1 cells and medium.

Statistical analysis

Mean values and standard deviations were computed using Excel (Microsoft, Redmond, WA). Significant differences were calculated by Student’s test.
and shown as *p < 0.05 and **p < 0.005. A p value <0.05 was deemed to be statistically significant.

Results

Suppression of human cell engraftment by the CD11c+B220+ pDC fraction

To investigate the effects of DCs on xenograft rejection, we first isolated two distinct DC fractions, CD11c+B220+ (pDCs) and CD11c+B220− (mDCs), from NOD-scid and NOG mice, respectively. These two DC fractions were intravenously transplanted into NOG mice before hPBMC transplantation (Fig. 1A). The efficacy of successful hPBMC engraftment in PB, BM, and spleens of NOG DC-transplanted NOG mice was at the same level as that of non-transplanted control NOG mice. In contrast, hPBMC engraftment was completely suppressed in the PB, BM, and spleens of NOD-scid pDC-transplanted NOG mice at 7 wk posttransplantation (Fig. 1B). Spleen cells from the NOD-scid, NOD-scid pDC-transplanted or mDC-transplanted NOG, and untreated NOG mice without hPBMC transplantation were cocultured with Yac-1 target cells that were labeled with [51Cr], then [51Cr] release into the culture supernatants was measured. Although cytotoxicity was less effective compared with spleen cells of NOD-scid mice, those from the pDC-transplanted NOG mice showed a higher level of cytotoxicity compared with mDC-transplanted NOG and non-transplanted NOG mice (Fig. 1C). These results show that the high level of engraftment of NOG mice is suppressed by the NOD-scid pDC fraction, indicating that this fraction includes cells that mediate xenograft rejection.

NK marker-expressed cells in the pDC fraction have the potential for xenograft rejection

To identify cells that play critical roles in graft rejection, we used NOD-scid mice that systemically expressed GFP (NOD-scid EGFP Tg), and transplanted the pDC and mDC fractions isolated from the mice into NOG mice before hPBMC transplantation. At 7 wk posttransplantation, engraftment of hCD45+ cells was inhibited in BM and spleens of the pDC-transplanted NOG mice (Fig. 2A, top left panel), consistent with the results shown in Fig. 1B. Also, GFP+ cells were detected in the spleens of the pDC-transplanted NOG mice but not in those of the mDC-transplanted NOG mice (Fig. 2A, top right panel), and these GFP+ cells expressed the DX5 Ag (Fig. 2A, bottom panel). These GFP+DX5+ cells might be expanded by recognizing xenografts and are considered to be responsible for their rejection. CD11c+B220+DX5+ cells are known to produce high levels of IFN-γ upon stimulation (26, 27). We compared the levels of IFN-γ production of the isolated DC subpopulations from the spleen cells of NOD-scid and NOG mice. After stimulation with either IL-12 plus IL-18 or PMA plus ionomycin, high-level production of IFN-γ was observed in the pDCs from the NOD-scid mice but not in those from the NOG mice (Fig. 2B). We compared the expression of Siglec-H, PDCA-1, and CD122 on CD11c+B220+ cells between NOD-scid and NOG mice. pDCs generally express Siglec-H and PDCA-1 (28, 29) but not CD122, which is an NK cell marker and often used for detection of DX5 (30). Siglec-H and PDCA-1 were expressed on all CD11c+B220+ cells in the spleen of NOG mice, but CD122+ cells were not observed (Fig. 2C). However, ∼30% of CD11c+B220+CD122+ cells were present in the spleens of NOD-scid...
mice. We further analyzed Ly49D expression on CD11c+B220+ CD122+ cells in NOD-scid mice. Ly49D is a receptor on NK cells and mediates allo rejection by recognition of MHC class I molecules (31, 32). The frequency of Ly49D expression on CD11c+B220+CD122+ cells was higher compared with that on CD11c+B220+CD122+ NK cells in the spleens of NOD-scid mice (Fig. 2D). These results suggest that CD11c+B220+CD122+ cells have a high potency for xenograft rejection and their absence in NOG mice may lead to high-level engraftment of human cells.

**CD11c+B220+CD122+ cells suppress xenograft rejection in NOG mice**

To determine whether CD11c+B220+CD122+ cells inhibit the engraftment of human cells, we isolated CD11c+B220+CD122+ cells or pDCs (CD11c+B220+CD122+) from NOD-scid mice by cell sorting (Fig. 3A). It is well known that pDCs produce type I IFNs after treatment with a TLR9 ligand (25). We demonstrated that isolated pDCs could produce IFN-α; however, IFN-α was not produced by CD11c+B220+CD122+ cells (Supplemental Fig. 1). CD11c+B220+CD122+ cells or pDCs were transplanted into NOG mice prior to hPBMC transplantation (Fig. 3A). Engraftment of the hCD45+ leukocytes was significantly suppressed in the PB, BM, and spleens of CD11c+B220+CD122+ cell-transplanted NOG mice, whereas the pDC-transplanted NOG mice showed similar percentages of engrafted human leukocytes to the non-transplanted control NOG mice at 7 wk posttransplantation with hPBMCs (Fig. 3B). We also isolated pDCs or CD11c+B220+CD122+ cells from NOD-scid and NOD-scid IFN-γ KO mice and transplanted them into NOG mice. The percentages of engrafted human leukocytes in the PB, BM, and spleens were not reduced by CD11c+B220+CD122+ cells from NOD-scid IFN-γ KO mice (Fig. 3C). These results revealed that CD11c+B220+CD122+ cells play a crucial role in xenograft rejection via IFN-γ production.

**Suppressive effects of CD11c+B220+CD122+ cells and NK cells on xenograft rejection**

The possible involvement of NK cells in xenograft rejection was examined because these cells produce IFN-γ and are defective in NOG mice. Thus, we compared the efficiencies of xenograft rejection for CD11c+B220+CD122+ cells and CD11c+B220+CD122+ NK cells. CD11c+B220+CD122+ cells and NK cells were sorted from the spleen cells of NOD-scid mice and intravenously transplanted (1 × 10⁵ or 2 × 10⁵ cells) into NOG mice before hPBMC transplantation (Fig. 4A). At 2 and 4 wk posttransplantation, the CD11c+B220+CD122+ cells were found to suppress human cell engraftment more potently than NK cells when 2 × 10⁵ cells were transplanted (Fig. 4B, right panel), although the CD11c+B220+CD122+ cells and NK cells did not cause rejection when 1 × 10⁵ cells were transplanted (Fig. 4B, left panel). In vitro cytotoxic assays showed that the killing activity of CD11c+B220+CD122+ cells was slightly higher than that of NK cells and that it could be suppressed by treating cells with an anti-NKG2D blocking Ab (Supplemental Fig. 2). Overall, these results indicate that CD11c+B220+CD122+ cells have a greater potential than NK cells to induce xenograft rejection.

**CD11c+B220+CD122+ cells are distinguishable from NK cells**

Previously, Vosshenrich et al. (33) reported that B220 expression on NK cells was inducible after activation in vitro and in vivo, and they argued that activated NK cells show a phenotypic resemblance to the CD11c+B220+CD122+ IFN-γ-producing killer dendritic cells (iKDCs). In contrast, Guimont-Desrochers et al. (34) showed that NK cells did not acquire B220 expression after adoptive transfer. To clarify this inconsistency, we investigated whether the sorted NK cells upregulated B220 and CD11c molecules after adoptive transfer. Fig. 5A shows that transplanted CD122+ cells did not acquire B220 expression in the spleens of NK cell-transplanted NOG mice, whereas a small amount of
FIGURE 3. CD11c+B220+CD122+ cells, but not pDCs, suppress xenograftment. (A) Scheme for the isolation of pDCs or CD11c+B220+CD122+ cells using a MoFlo cell sorter and subsequent cell transfer (2 × 10^5 cells) into NOG mice before transplantation of hPBMCs (5 × 10^6 cells). (B) hCD45+ human cell engraftment was analyzed by flow cytometry in the PB, BM, and spleens at 7 wk after hPBMC transplantation. The pDC-transplanted NOG mice (n = 5) showed a similar engraftment rate to that of the non-treated NOG (n = 3) mice, whereas a remarkable decrease in human cell engraftment was observed in the CD11c+B220+CD122+ cell-transplanted NOG mice (n = 5). (C) CD11c+B220+CD122+ cells from NOD-scid or NOD-scid IFN-γ KO mice were isolated and transplanted (2 × 10^6 cells) into NOG mice, followed by transplantation of 5 × 10^6 hPBMCs. The percentages of engrafted leukocytes were analyzed by flow cytometry at 6 wk post-transplantation. The percentage of hCD45+ cells was inhibited in the PB, BM, and spleens of the NOD-scid CD11c+B220+CD122+ cell-transplanted NOG mice (n = 11), whereas IFN-γ KO CD11c+B220+CD122+ cells did not inhibit hCD45+ cells as well as nontransplanted control NOG mice (n = 6). **p < 0.005.

FIGURE 4. Suppressive effects of CD11c+B220+CD122+ and NK cells on xenograftment. (A) CD11c+B220+CD122+ cells or NK cells were positively isolated from the CD11c+B220+ fraction or CD11c-B220- fraction of NOD-scid mice spleen cells using the MoFlo cell sorter, and 1 × 10^5 or 2 × 10^5 cells were transplanted into NOG mice before hPBMC transplantation. (B) At 2 and 4 wk posttransplantation, RBC-lysed PBMCs were collected from the transplanted NOG mice, and the efficacy of human cell engraftment was analyzed by flow cytometry. The percentage of engrafted hCD45+ cells was significantly lower in the 2 × 10^5 CD11c+B220+CD122+ cell-transplanted NOG mice (n = 6) than in the 2 × 10^5 NK cell-transplanted mice (n = 7). *p < 0.05, **p < 0.005.

CD122+ cells simultaneously expressed B220 and CD11c molecules in the spleens of CD11c+B220+CD122+ cell-transplanted NOG mice. We further analyzed the morphological differences among sorted CD11c+B220+CD122+ cells, NK cells, and pDCs by May–Giemsa staining. As observed in Fig. 5B, CD11c+B220+CD122+ cells resembled pDCs; they showed a monocytic mor-
phology, low nuclear/cytoplasmic ratio, and dispersed chromatin. In contrast, NK cells showed a lymphocytic morphology, high nuclear/cytoplasmic ratio, and hyperchromatic nuclei. Moreover, we compared the area and perimeters of these cell populations using ImageJ analysis. CD11c+B220+CD122+ cells had an intermediate area size between that of pDCs and NK cells and a larger perimeter than NK cells (Fig. 5C). These results demonstrated that CD11c+B220+CD122+ cells were phenotypically and morphologically distinct from NK cells.

Discussion

In the current study, we investigated the mechanism underlying the high acceptance rate of xenografts in NOG mice. To determine the cells responsible for xenograft rejection, we transplanted DC subpopulations and NK cells from NOD-scid mice into NOG mice. We showed that the CD11c+B220+CD122+ cells from NOD-scid mice strongly inhibited the engraftment of transplanted hPBMCs, whereas other DC subpopulations and NK cells from NOD-scid mice did not highly contribute to xenograft rejection in NOG mice. Throughout these experiments, we revealed that CD11c+B220+CD122+ cells were phenotypically and morphologically distinct from NK cells.

IKDCs and activated NK cells—which are controversial cell lineages in xenograft rejection—are responsible for the rejection in immunodeficient mice.

A DC subpopulation that expresses NK cell markers CD122 and DX5 in the pDC fraction was identified as IKDCs, which share the functional properties and surface markers of DCs and NK cells and have cytotoxic activities and Ag-presenting abilities (35, 36). Taieb et al. (36) reported that IKDCs prevented transplanted tumor outgrowth and that IFN-γ—dependent and TRAIL-dependent killing activities and tumor recognition via MHC class II molecules occurred simultaneously when these cells were adoptively transferred, whereas conventional NK cells did not prevent these events. The authors claimed that IKDCs participate in tumor surveillance and act as effectors of innate immune responses. Regarding activated NK cells, CD11c+B220+CD122+ cells more closely resemble NK cells than DCs (33, 37–39) because their developmental pathway is very similar to that of NK cells that rely on IL-15 signaling through the IL-2Rβ, IL-15Rβ, and common γ-chain. In our results, the CD11c+B220+CD122+ cells resembled activated NK cells but not pDCs, as CD11c+B220+CD122+ cells did not express Siglec-H and PDCA-1 and did not produce IFN-α.
after TLR9 stimulation, and cytotoxicity was suppressed by NK2D Ab treatment. These results were consistent with activated NK cells reported by Blasius et al. (39). However, CD11c<sup>B220</sup>CD122<sup>+</sup> cells were morphologically distinct from NK cells, and NK cells did not acquire B220 expression after adaptive transfer. Recently, Guimont-Desrochers et al. (34) obtained similar results demonstrating that CD11c<sup>B220</sup>CD122<sup>+</sup> cells are distinct from activated NK cells. Thus, controversy remains in describing the relationship between CD11c<sup>B220</sup>CD122<sup>+</sup> cells and activated NK cells, and further studies are needed to define the differences between these cells.

The role of NK cells in xenograft rejection in NOD-<i>scid</i> mice has been described previously. Kollet et al. (15) reported an 11-fold higher rate of xenograftment after transplantation of human HSCs for NOD-<i>scid</i> <i>β2m<sup>null</sup></i> mice that lacked NK activity compared with that for NOD-<i>scid</i> mice. Additionally, McKenzie et al. (17) showed enhancement of xenograftment after HSC transplantation when the NK cells were eliminated by treatment with a CD122 Ab. In those studies, the observed higher xenograftment level cannot be attributed solely to NK cells because CD11c<sup>B220</sup>CD122<sup>+</sup> cells could have been present in their eliminated fraction. Our current results indicate that CD11c<sup>B220</sup>CD122<sup>+</sup> cells produce higher levels of IFN-γ than NK cells upon IL-15 stimulation (35, 36). Chaudhry et al. (40) reported that CD11c<sup>B220</sup>NK1.1<sup>+</sup> cells (called NKDCs), which are the same as CD11c<sup>B220</sup>CD122<sup>+</sup> cells, and NK cells were absent in IL-15<sup>−/−</sup> mice, and they observed that CD11c<sup>B220</sup>NK1.1<sup>+</sup> cells were restored more rapidly than NK cells by exogenous IL-15 treatment. Their in vitro analysis showed that CD11c<sup>B220</sup>NK1.1<sup>+</sup> cells stimulated with IL-15 retained cytotoxic capacity and potent IFN-γ secretion. Moreover, tumor metastasis in the lung caused by transplanted B16F10 melanoma cells was inhibited by syngeneic CD11c<sup>B220</sup>NK1.1<sup>+</sup> cell transplantation. In contrast, CD11c<sup>B220</sup>NK1.1<sup>+</sup> cells from IFN-γ-deficient mice did not cause this type of inhibition. Furthermore, Lin et al. (41) transplanted pig cells into T cell-depleted, IFN-γ-deficient mice and found that the engraftment of pig cells was significantly enhanced in the IFN-γ-deficient mice compared with the control T cell-depleted, wild-type mice. Those findings support our current results that CD11c<sup>B220</sup>CD122<sup>+</sup> cells from IFN-γ-deficient mice do not suppress xenograftment. Thus, xenograft rejection by CD11c<sup>B220</sup>CD122<sup>+</sup> cells may depend on the amount of IFN-γ, and IFN-γ deficiency may contribute to the high acceptance rate of xenografts in NOG mice. However, we have no evidence that the IFN-γ produced by CD11c<sup>B220</sup>CD122<sup>+</sup> cells modulates the rejection of xenografts. We speculate that IFN-γ can promote xenograft rejection through at least two scenarios. In the first scenario, CD11c<sup>B220</sup>CD122<sup>+</sup> cells self-activate in an autocrine manner through secretion of IFN-γ and enhance cytotoxicity mediated by perforin/granzyme, FasL, and the TRAIL pathway against targeted xenografts that lack species-specific MHC class I molecules, which interact with killer inhibitory receptors (42). In the second scenario, IFN-γ produced by CD11c<sup>B220</sup>CD122<sup>+</sup> cells induces the activation of macrophages, and these macrophages are subsequently recruited to the graft site through the upregulation of monocyte-activating chemokines, resulting in a direct attack on the xenograft. The relative importance of IFN-γ-dependent xenograft rejection by CD11c<sup>B220</sup>CD122<sup>+</sup> cells remains to be elucidated.

In conclusion, we demonstrated that the high-level acceptance of xenografts in NOG mice is due to a lack of CD11c<sup>B220</sup>CD122<sup>+</sup> cells, and we suggest that IFN-γ produced by CD11c<sup>B220</sup>CD122<sup>+</sup> cells plays an important role in xenograft rejection. These data are useful for clarifying the immunological mechanisms leading to rejection of xenotransplants. Further studies are needed to confirm the exact pathway involved in CD11c<sup>B220</sup>CD122<sup>+</sup> cell-dependent mechanisms of xenograft rejection.

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Disclosures

The authors have no financial conflicts of interest.
Supplementary figure legends

Figure S1. CD11c- B220+ CD122+ cells do not produce IFNα.

CD11c+ B220- CD122+ cells or CD11c+ B220+ CD122- pDCs were isolated from the CD11c+ B220+ fraction or CD11c- B220- fraction of NOD-scid mouse spleen cells using the MoFlo cell sorter. These fractions were cultured in RPMI 1640 medium (Invitrogen) containing 10% FCS in 96-well flat-bottomed plates at 37°C in 5% CO₂. To induce IFNα, cells were stimulated with 1μM CpG ODN 2216 (Sigma-Aldrich) for 48 h. Culture supernatants were collected and IFNα was assayed using the Verikine Mouse IFNα ELISA Kit (PBL Interferon Sources, Piscataway NJ).

Figure S2. Cytotoxicity is reduced via inhibition of an NKG2D receptor on CD11c+ B220+ CD122+ and NK cells.

Non-RI cytotoxic assay was performed using the Cellular DNA Fragmentation ELISA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacture’s instructions. Sorted CD11c+ B220- CD122+ or NK cells from the spleens of NOD-scid mice were stained with anti-mouse NKG2D and isotype control antibody (Biolegend), respectively, and co-cultured with BrdU-labeled Yac-1 cells at various ratios for 4 h. After co-culturing, the supernatant was removed and amount of BrdU-labeled fragmented DNA was measured by ELISA. The percentage of lysis was calculated as described in Cytotoxicity measurements in the Materials and Methods.