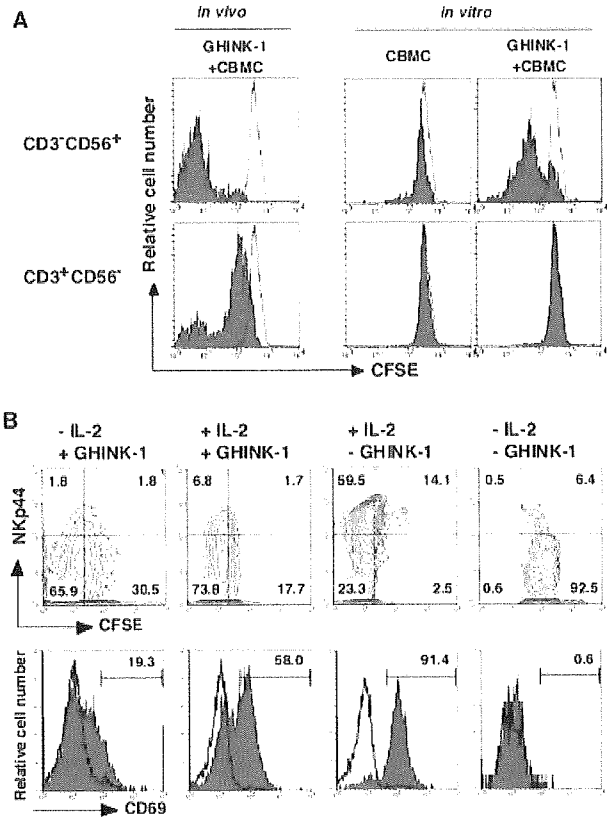

Figure 3. GHINK-1 cells induce selective proliferation of human NK cells *in vitro* and *in vivo* without exogenous IL-2. (A) CFSE-labeled CBMC were intraperitoneally injected with GHINK-1 cells into NOD/SCID mice, or cultured with or without GHINK-1 cells *in vitro*. After 7 days, the cells were harvested and stained with anti-CD56-PE and CD3-PC5 mAb, and fluorescence intensity of CFSE was analyzed on $CD3^-CD56^+$ and $CD3^+CD56^-$ cells. Open profiles indicate non-divided cells. (B) CFSE-labeled or unlabeled CBMC was co-cultured under indicated conditions. Cells were stained with anti-CD3-PE/Cy7, CD56-PC5 and NKp44-PE, or anti-CD69-FITC, CD3-APC and CD56-PE8. CFSE reduction and expression of NKp44 and CD69 on $CD3^-CD56^+$ NK cells were analyzed by flow cytometry at day 5. Numbers indicated percentage of the cells in each fraction. One set of representative results from three independent experiments is shown.

respectively), while there was no significant difference between GHINK-1 -transplanted and CBMC-transplanted mice ($p=0.5$) (Fig. 7A). Subsequently, we performed immunohistochemistry to detect $CD16^+$ cells in the tumor tissues of the co-transplanted mice. $CD16^+$ cells were observed in the tumor tissues, particularly, around necrotic tumor cells (Fig. 7B). These results suggested that the human NK cells migrated to tumor cells and killed them *in vivo*.

Discussion

In this study, we established a mouse model that engrafted human NK cells. Human NK cells were selectively generated in NOD/SCID mice with intraperitoneal co-transplantation of CBMC and GHINK-1, a cell line that selectively stimulates human NK cell proliferation. Two human NK cell populations, cytotoxic $CD56^{dim}CD16^+$ and immature $CD56^-CD16^+$ cells, appeared in PB and other hematopoietic organs of the mice. Most of the NK cells generated did not express activation markers (CD25 and CD69) or the activated NK cell marker (NKp44), indicating that they are resting NK cells resembling human PB and CB NK cells. These NK cells were activated by stimulation with IL-2, and showed antitumor effects both *in vivo* and *in vitro*. Thus, the NK cells generated in this system are functionally and phenotypically identical to those in human PB. The mouse model provides information about the dynamics of physiological NK cells *in vivo* and an important preclinical evaluation system for immunotherapeutic strategies.

Our understanding of the development and function of human NK cells is largely based on *in vitro* analysis, and appropriate models to study human NK cells *in vivo* have been lacking. The NOD/SCID mouse was found to be an efficient recipient for the reconstitution of human hematopoietic cells. However, the lymphoid differentiation is restricted to the B cell lineage [13], and T and NK cells are produced at a minimum level even in modified strains like NOD/SCID β2-microglobulin-deficient [14] or NOD/SCID common γc-deficient mice [18]. Recently, Kalberer *et al.* [20] reported engraftment of human NK cells on administration of IL-15 and Flt-3 ligand into $CD34^+$ cell-transplanted NOD/SCID mice. In their model, the administration of these growth factors specifically generated mature $CD56^+$ NK cells and $CD34^-CD7^+$ NK precursor cells in bone marrow and spleen over a long period (more than 5 months). A number of studies have now demonstrated that human NK cells can be generated *in vitro* from $CD34^+$ hematopoietic stem cells in cultures that contain key cytokines (IL-2, IL-12, IL-15 etc.) and multi-lineage hematopoietic growth factors (SCF and Flt-3 ligand) [21–24]. On the other hand, stroma cells are said to play an important role in NK cell development [41]. Although some stroma cell molecules can be replaced by cytokines such as SCF, Flt3-ligand and IL-15 [21–24], we have recently demonstrated that a Wilms' tumor cell line, HFWT (original strain of GHINK-1 cells), can stimulate the differentiation of mature NK cells from Lin^-CD122^+ NK precursors, but not from $CD34^+$ hematopoietic stem cells [34]. Thus, cell-to-cell interaction is essential for the generation of NK cells and soluble factors such as IL-15 are not involved in this process [29]. This represents

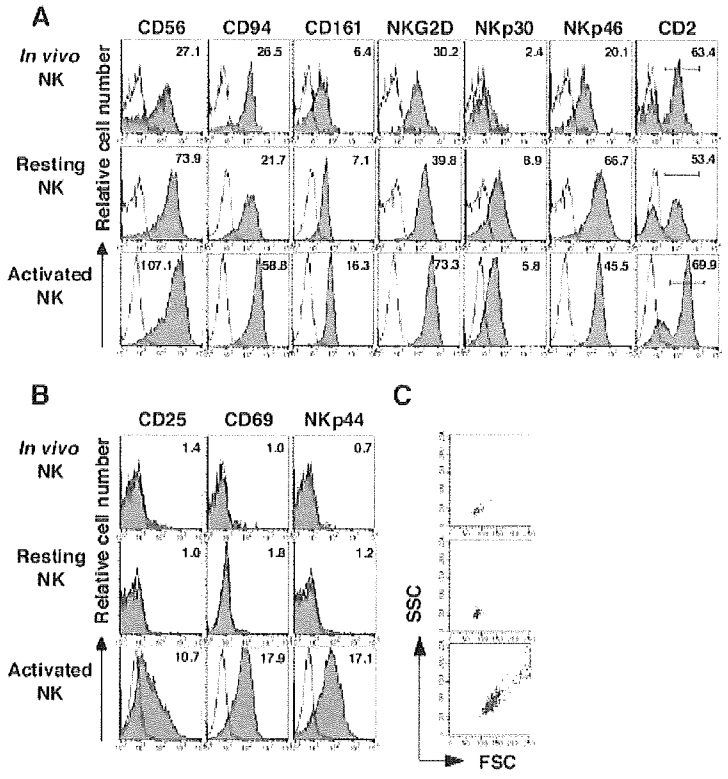

Figure 4. Surface antigen expression on the NK cells generated in NOD/SCID mice. NK cells generated in NOD/SCID mice (day 12 after the co-transplantation) (*In vivo* NK), freshly isolated CBMC (Resting NK), and CBMC cultured *in vitro* with GHINK-1 cells and IL-2 for 10–14 days (Activated NK) were stained with the indicated mAb and analyzed on $CD16^+$ cells by flow cytometry. (A) CD94, CD161, NKG2D, NKp30, NKp46 and CD2 are the NK cell receptors and costimulatory molecule, respectively. Numbers in CD2 show percentages of positive cells, and others show relative mean fluorescence intensity (monoclonal antibody/negative control). (B) CD25, CD69 and NKp44 are activation markers. Open profiles indicate negative control. (C) FSC/SSC dot plot indicates cell size and granularity. One set of representative results from three independent experiments is shown.

the phenotypic differences of the mice. In the study of Kalberer *et al.*, cytokine-induced NK cells mainly consist of $CD56^+CD16^+$ cells that express the activation marker CD69. In contrast, our GHINK-1-generated NK cells are $CD56^{dim}CD16^+$ and $CD56^-CD16^+$ NK cells with the resting phenotype. This discrepancy can be explained by the notion that IL-15 works as a potent activator of NK cells as well as NK cell development, whereas cell-to-cell contact may provide both proliferative signals and inhibitory signals for activation. This is in agreement with the fact that hematopoietic stem cells proliferate but differentiate in the presence of soluble cytokines, while they proliferate and self renew in the presence of stroma cells. Thus, stroma cells or the microenvironment appropriately regulate the development and function of hematopoietic cells through a balance of positive and negative signals. One important molecule that interacts between hematopoietic stem cells and stroma cells is CXCR4 and its ligand SDF-1 [42]. NK cells also express CXCR4 [43], and Beider *et al.* [19] showed that CXCR4 is heavily involved in the homing and retention of human NK and NKT cells at the bone marrow and spleen of NOD/SCID mice. Following activation with IL-2, the levels of CXCR4 on NK and NKT cells decreased significantly, and inhibited the homing and retention in the bone marrow and spleen of NOD/SCID mice [19]. Thus, stroma cells regulate the development and function of NK cells. In this study, we revealed that GHINK-1 cells stimulate proliferation of resting NK cells without activation both *in vivo* and *in vitro* (Fig. 3, 4). Ferlazzo *et al.* [44] reported that human DC also stimulates proliferation of resting NK cells with slight increases of activating markers expression. It is possible that a putative ligand for resting NK cell stimulation expressed on DC is also expressed on GHINK-1 cells, and that the ligand on GHINK-1 cells reacts with unknown receptors selectively expressed on NK cells. In this context, the identification of responsible molecules on GHINK-1 feeder cells will enable us to clarify the regulatory mechanisms of NK cell development and function.

Human PBMC contain approximately 10% NK cells [1]. Subsets of NK cells in adult PBMC can be distinguished by the surface density of CD56 antigen as well as by the presence or absence of CD16 antigen. The majority are $CD56^{dim}CD16^+$, the most cytotoxic subset. A minor NK subset in PB is formed by $CD56^{bright}CD16^-$ cells, which produce a series of cytokines including IFN-γ, TNF-α, GM-CSF, and IL-10 upon activation, while $CD56^{dim}CD16^+$ NK cells produce few of these cytokines [5]. There are few $CD56^-CD16^+$ NK cells in adults, but they have been identified in CB as possible precursors of mature $CD56^+$ NK cells, which, when cultured in the presence of IL-2, IL-12, or IL-15, acquire adult-like cytotoxic activity and cytokine production [33, 34]. The NK cells generated *in vivo*

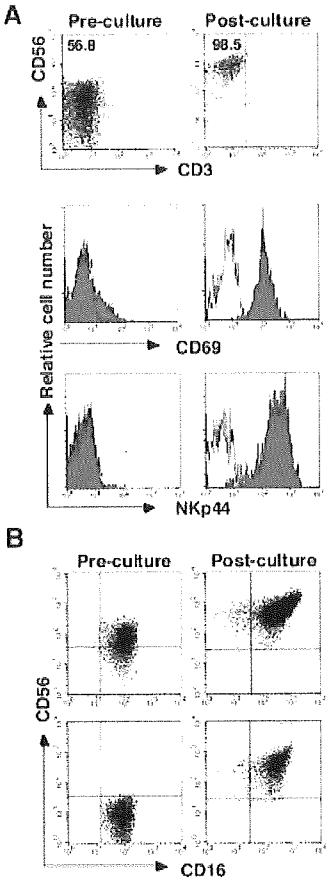

Figure 5. *In vitro* activation of human CD56^{dim}CD16^{+} and CD56^{-}CD16^{+} NK cells derived from NOD/SCID. Cells were harvested from the peritoneal cavity of NOD/SCID mice at day 12 after the co-transplantation, and investigated for the expression of cell surface markers. (A) Expression of CD3 and CD56 on CD45^{+} cells, and CD69 or NKp44 on CD16^{+} cells. Open profiles indicate the negative control. (B) Expression of CD56 on purified CD56^{dim}CD16^{+} and CD56^{-}CD16^{+} cells. Freshly isolated cells (Pre-culture, left panels) were cultured with IL-2 for 14 days (Post-culture, right panels). One set of representative results from three independent experiments is shown.

consisted of CD56^{dim}CD16^{+} and CD56^{-}CD16^{+} cells but not CD56^{bright}CD16^{-} NK cells in our model. In contrast, in the study of Kalberer *et al.*, the growth factor-treated mice predominantly generated CD56^{bright}CD16^{-} cells [20]. These results suggest that the development of CD56^{dim}CD16^{+} and CD56^{bright}CD16^{-} cells requires different molecules. It has previously been suggested that CD56^{bright}CD16^{-} cells are precursors of CD56^{dim}CD16^{+} cells [45], but the finding that our GHINK-1-treated mice had no CD56^{bright}CD16^{-} cells and that CD56^{-}CD16^{+} immature NK cells could differentiate into the CD56^{dim}CD16^{+} NK cells [34] (Fig. 5B) support that CD56^{bright}CD16^{-} cells are not the direct precursors of CD56^{dim}CD16^{+} NK cells. Thus, our model mice provide information about NK cell development as well

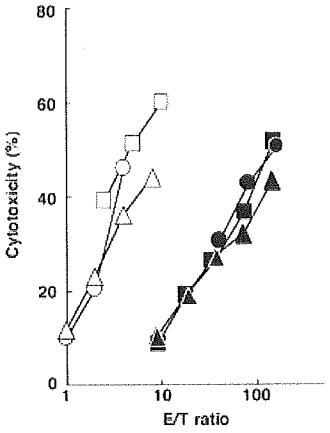

Figure 6. Cytotoxicity of human NK cells from NOD/SCID mice is augmented by IL-2. Cells were harvested from the peritoneal cavity of NOD/SCID mice at day 12 after the co-transplantation. They were assayed for cytotoxicity immediately (closed symbols), or after cultivation with IL-2 for 10–14 days (open symbols). The effector cells were co-cultured with EGFP-K562 target cells at the indicated E/T ratios. After 6-h incubation, PI was added to the cell mixture, and cytotoxicity was measured by flow cytometry. Cytotoxicity was calculated from percentages of PI^{+} and EGFP^{+} cells as described in Materials and Methods. Three different symbols represent three independent experiments from three donors.

as the function of the main NK population in human PB (CD56^{dim}CD16^{+}).

NK cells are innate immune lymphocytes, which showed early clinical promise because of their ability to lyse tumor cells without specific antigen recognition. Clinical trials attempting to harness the antitumor effect of NK cells, either through *in vivo* or *in vitro* activation [46–48], however, have met with only modest success to date. These trials were based on *in vitro* cytotoxicity or activated NK cell-mediated antitumor activity in the mouse model, and their experimental results did not directly link to clinical stages. The next immunotherapeutic strategies will be to modify resting NK cell functions. For example, chemokine gene transduction into tumor cells to enhance recruitment of NK cells or tumor-specific mAb treatment to induce antibody-dependent cellular cytotoxicity (ADCC), which is mediated through CD16 on cytotoxic CD56^{dim} NK cells may be a good candidate for immunotherapy using NK cells. However, such pre-clinical examinations could not be performed, because no suitable animal model is available that has steady-state "resting" human NK cells. In the present study, the human NK cells of NOD/SCID origin showed an identical phenotype to resting (blood circulating) CD56^{dim}CD16^{+} NK cell (Fig. 4) and cytotoxic activity *in vitro* (Fig. 6), and are capable of reducing K562 erythroleukemia tumor formation *in vivo* (Fig. 7). Therefore, our present model mouse has functional and physiologically resting NK cells. This

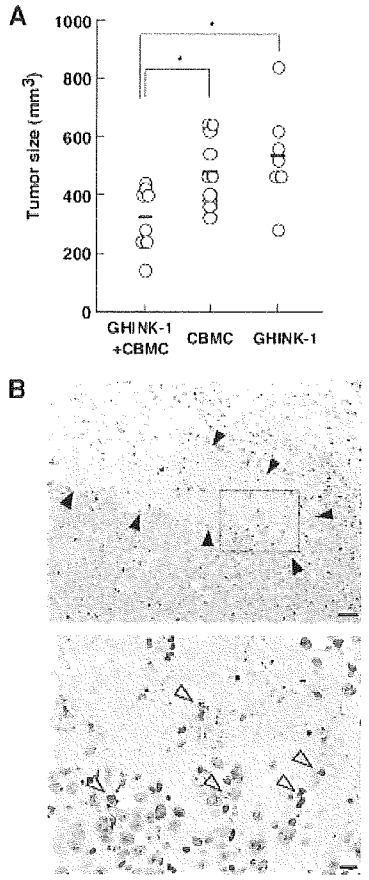

Figure 7. The NK cells generated in mice exhibit antitumor activity and infiltrate into the tumor tissues. (A) K562 cells were injected subcutaneously into the right flank of NOD/SCID mice. Eight days later, CBMC and GHINK-1, CBMC or GHINK-1 cells were injected into peritoneal cavity of the mice. Tumor size (mm^3) was measured at day 14 after the injection of K562 cells. Data for each group were obtained from seven to ten mice. (B) Human NK cells infiltrated K562 tumor tissue. CBMC and GHINK-1 cells were co-transplanted into NOD/SCID mice, and K562 cells were injected subcutaneously into the mice as described in Materials and Methods. After 14 days, the tumor tissues were subjected to immunohistochemistry with anti-CD16 mAb and visualized with 3,3′-diaminobenzidine. Regions within closed triangles are necrotic tumor tissue (upper panel). The lower panel shows a close-up of part of the upper panel. Open triangles show $CD16^+$ cells. Scales in upper and lower panels represent 40 and 10 μm, respectively.

model mouse will be useful for pre-clinical study of NK cell-based immunotherapies.

The translation of NK cell biology to the clinic should result in a significant improvement of NK-cell based therapies for cancer. Our mice represent an important model for evaluation of the immunotherapeutic efficacy of human NK cells and for investigations of the factors and signals orchestrating (coordinating) the formation of the NK cell compartment in humans.

Materials and methods

CB, cell lines and culture

CB was kindly provided by Fukuda Hospital (Kumamoto, Japan) with the informed consent of the mothers. Investigations were approved by the Ethical Committee of the Kumamoto University Graduate School of Medical Science. CBMC were separated by LSM lymphocyte separation medium (Cappel, Aurora, OH) density-gradient centrifugation for use in the *in vitro* and *in vivo* experiments.

A subline of the Wilms' tumor cell line HFWT, GHINK-1, was established and maintained in DMEM (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated FBS [30]. The K562 cell line was obtained from RIKEN Cell Bank (Tsukuba, Japan). EGFP-transfected K562 (EGFP-K562) cells were established with pEGFP-N1 (BD Biosciences, San Diego, CA) and the transfection reagent Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's instruction manual. Original and EGFP-K562 cell lines were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FBS.

CBMC were cultured with or without irradiated GHINK-1 cells in RPMI 1640 medium (Sigma) supplemented with 10% autologous plasma without IL-2 (Shionogi Chemical Institute, Osaka, Japan) for 7 days. Engrafted human cells in the peritoneal cavity of NOD/SCID mice were cultured in the above medium with 200 U/mL IL-2 for 10–14 days. Activated NK cells were obtained by culturing CBMC and irradiated (50 Gy) GHINK-1 cells with 200 U/mL of IL-2 for 10–14 days as previously described [27].

Mice and transplantation

NOD/SCID mice purchased from Clea Japan (Tokyo, Japan) were bred and maintained in laminar flow housing at the Center for Animal Resources and Development (CARD), Kumamoto University. NOD/SCID mice, 8–12 weeks old, were irradiated (2.5 Gy) and 3×10^7 CBMC with or without 3×10^6 irradiated (50 Gy) GHINK-1 cells were injected intraperitoneally. After transplantation, leukocytes were harvested from PB, peritoneal cavity, spleen, bone marrow and liver for further analysis. Red blood cells were lysed with red cell lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA). In the case of liver, homogenized specimens were passed through a cell strainer (70 μm) and mononuclear cells were obtained by density-gradient centrifugation as described above. All of the animal experiments were performed according to the guidelines of Kumamoto University, Graduate School of Medical Science.

Flow cytometry and cell sorting

FITC-labeled anti-CD16 (3G8), -CD69 (FN50), -CD94 (HP-3D9), -CD161 (VI NK12), -DNAM-1 (DX11), -NKB1 (DX9), PE-labeled anti-CD7 (M-T701), -CD56 (B159) and -CD25 (M-A251) were purchased from BD PharMingen (San Diego, CA). FITC-labeled anti-CD2 (G11), -CD3 (S4.1) and PE-Cy5 (PC5)-labeled anti-CD45 (HI30) were provided by Caltag (Burlingame, CA). PE-labeled anti-CD11a (25.3), -NKG2D (ON72), -NKp30 (Z25), -NKp44 (Z231), -NKp46 (BAB281) and PC5-

labeled anti-CD3 were obtained from Beckman Coulter (Fullerton, CA). Isotype-matched control mAb were used as negative controls. Cells were treated with red cell lysing buffer to lyse erythrocytes before staining. Single-cell suspensions were prepared in staining medium (PBS with 3% FBS and 0.05% sodium azide), and stained with the mAb described above. After a 30-min incubation on ice, cells were washed twice, resuspended in staining medium, and immediately analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cell proliferation was observed using CFSE fluorescence labeling reagent (Molecular Probes, OR). Staining was conducted according to the manufacture's instructions. CFSE-labeled CBMC were further stained with anti-CD56-PE and anti-CD3-PC5 mAb as described above. $CD45^+CD56^{-\text{ or dim}}$ $CD16^+$ cells from the peritoneal cavity of the mice were sorted on a JSAN flow cytometer (Bay Bioscience, Japan).

Cytotoxicity assay

The cytotoxic activity of the NK cells was measured by flow cytometry with EGFP-K562 cells as described by Kantakamalakul *et al.* [40]. Briefly, 50-μL aliquots of EGFP-K562 cells (1×10^5 cell/mL) were placed in 5-mL round-bottom tubes, and 400 μL twofold serial diluted effector cells was added. Effector alone was used as a negative control. After a 6-h incubation at 37°C, 50 μL 20 μg/mL propidium iodide (PI) was added with incubation for an additional 15 min. Target cells in FSC (forward scatter) *vs.* SSC (side scatter) dot plots were gated, and GFP and PI were measured by FACScan. Cytotoxic activity was calculated as follows: $[A/(A+B) \times 100] - C$ (%), where A is the percentage of PI^+EGFP^+ cells; B is the percentage of PI^-GFP^+ cells at each E/T ratio; C is the percentage of spontaneous PI^+ cells without effector cells $[A/(A+B) \times 100$ (%) at E/T ratio $=0]$.

Antitumor activity *in vivo* (K562 tumor formation)

K562 cells (1×10^6 cells/mouse) were transplanted subcutaneously into the right flank of the NOD/SCID mice at day 8 after the intraperitoneal injection of CBMC and GHINK-1 cells, CBMC alone, or GHINK-1 cells alone. Tumor growth was monitored by measuring maximal and minimal diameters with calipers every 3–4 days, and tumor size was estimated with the formula: tumor size (mm^3) = length (mm) × $width^2$ (mm) × 0.4 as described previously [49]. The effect of NK cells on tumor growth was determined in groups of seven to ten mice. For immunohistochemistry, CBMC with or without GHINK-1 cells were injected simultaneously with the subcutaneous injection of K562 cells. After 14 days, the tumor tissues were subjected to immunohistochemistry.

Immunohistochemistry

Tumor tissues were removed from the mice and fixed with 10% formalin. The tumor specimens were embedded in paraffin. Paraffin sections, 4 μm thick, were deparaffinized through graded ethanol and rehydrated in distilled water. Endogenous peroxidase was inhibited by a 30-min incubation in methanol containing 0.3% H_2O_2. To optimize immunodetection, nonenzymatic antigen unmasking was performed by heating tissue sections for 10 min in a microwave in 5 mM citrate buffer (pH 6). After cooling, sections were incubated with normal goat serum (Nichirei, Tokyo, Japan) diluted in PBS containing 1% BSA for 30 min. Incubation with the anti-CD16 (2H7) mAb (Novocastra, Newcastle-upon-Tyne, UK) was performed overnight at 4°C. Sections were subsequently rinsed three times in PBS with Triton X-100, and treated with biotinylated goat anti-mouse immunoglobulin (Nichirei) for CD16 staining. After rinsing, the sections were incubated with streptavidin-horseradish peroxidase complex (Nichirei) for 30 min and finally visualized with the use of 3,3′-diaminobenzidine (DAKO, Glostrup, Denmark) in 0.05 M acetate buffer containing 0.015% H_2O_2. The slides were counterstained with hematoxylin. As positive control for CD16 immunostaining, paraffin sections of human tonsil were processed using the same immunostaining procedures, and as a negative control, the same staining method was carried out without the antisera.

Statistical analysis

The statistical significance of differences was determined using Student's *t*-test. *p* values less than 0.05 were defined as statistically significant.

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