Antigen-Specific Antibody Production of Human B Cells in NOG Mice Reconstituted with the Human Immune System

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Abstract Passive antibody administration shows strong potential as a new therapeutic method. In clinical applications, human-derived antibodies with antigen specificity are more useful without putting individuals at risk. Production of human-derived antibodies against given antigens can be obtained from animal models if the human immune system is established in the animals. In fact, past reports revealed that human T and B cells develop from hematopoietic progenitor cells in immunodeficient mice. However, there have been few reports on sufficient induction of antigen-specific antibodies, particularly IgG, in immunodeficient mice reconstituted with human immune cells. In this chapter, we discuss a major

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T. Nomura et al. (eds.), *Humanized Mice.*
Current Topics in Microbiology and Immunology 324.

shortcoming of induction of antigen-specific IgG antibodies in human immune cells developed in the murine environment based on our data. We demonstrated that human T cell development is restricted by the murine MHC and consequently T cells may not achieve cognate interaction with human B cells. Human B cells developed in the mouse are mainly CD5+B1 cells that preferentially produce IgM. At the same time, human LN transplantation on the spleen enabled NOG mice to produce antigen-specific IgG antibody. These results suggest that if efficient cognate interaction mediated by a certain antigen on MHC class II between human T and B-2 cells occurs, human B cells can produce IgG antibody against a given antigen in the murine environment.

Abbreviations APC: antigen-presenting cell; BM: bone marrow; CB: cord blood; DC: dendritic cell; DN: double negative; DNP-KLH: 2,4-dinitrophenylated keyhole limpet hemocyanin; DP: double positive; ELISA: enzyme-linked immunosorbent assay; FACS: fluorescence-activated cell sorting; GVHD: graft-versus-host disease; Ig: immunoglobulin; IL: interleukin; KO: knockout; LN: lymph node; mAb: monoclonal antibody; MACS: magnetic cell sorting; MHC: major histocompatibility complex; NK: natural killer; NOD: nonobese diabetic; NOG: NOD/Shi scid IL2R gamma chain knockout; OVA: ovalbumin; PBL: peripheral blood lymphocyte; PMA: phorbol 12-myristate 13-acetate; RTOC: reaggregate thymic organ culture; SCID: severe combined immunodeficiency; TSST-1: toxic shock syndrome toxin-1; TCR: T cell receptor; WT: wild type

1 Introduction

Reports showing the therapeutic effect of passive antibody administration in some diseases such as cancer and autoimmune diseases are increasing [4, 19, 24]. To date, most antibodies used for clinical application have been derived from mouse monoclonal antibodies (mAb) and humanization of the mAb has been performed with genetic engineering techniques. However, it is still uncertain whether patients who receive such humanized mAb suffer from certain harmful effects because they may produce their own antibody against administered mAb even if mAb are partially or almost completely humanized. Currently, some mutant mice have been highlighted as a tool for generating less risky mAb. Since these mutant lines carry human chromosome fragments containing immunoglobulin gene clusters [26], the gene product is identical to the human product. However, it is still possible that patients may produce antibody against mAb obtained from such mutant mice because their immunoglobulin contains murine-origin sugar components [15]. If we can develop a laboratory animal in which human-derived B cells can produce antibodies against given antigens, the antibodies should be extremely safe for clinical application. In the 1990s, investigators attempted to reconstitute human-origin hematopoiesis and/or lymphopoiesis

by using severe combined immunodeficiency (SCID) mice that can receive xenografts because of their lack of T and B cell development [12, 16]. In those studies, however, human T cells did not develop in the mice if the human fetal thymus was not simultaneously engrafted [14,17] despite the fact that human T cells develop from cord blood (CB) or bone marrow (BM) cells in organ cultures of murine thymus lobes [20, 21, 28]. After other SCID background lines with no natural killer (NK) cells (NOD-SCID-IL-2Rg−/−, abbreviated as NOG mice) or with reduced NK activity (NOD-SCID mice) became available, in vivo development of human T [11, 27] and B [8, 13] cells from human CB and BM cells was reported in the mouse. Because of their remarkably high efficiency for normal human cell engraftment including CB cells [7], we have used NOG mice reconstituted with human CD34+CB cells (abbreviated as CB-NOG mice) to induce antigen-specific antibody derived from human B cells after immunization. Here, based on new and previous data concerning development of human T and B cells in NOG mice, we discuss why antigen-specific IgG antibody is almost undetectable in mice reconstituted with human CD34+ cells.

2 Antibody Production in the CB-NOG Mouse After Immunization

2.1 Past Studies on Human Antibody Production in Immunodeficient Mice

Initial studies attempted to induce antibody production from human B cells in immunodeficient mice such as SCID or NOD-SCID mice implanted with human peripheral blood lymphocytes (PBL) containing mature T and B cells [1, 16, 22]. However, these attempts were almost all unsuccessful, presumably because graft-versus-host diseases (GVHD) occurred because of the presence of human immune cells in the recipient mice. To avoid GVHD the engrafted PBL number was decreased and the problem of GVHD was basically eliminated, but the antigen-specific antibody became almost undetectable, presumably because human immune cells are dispersed at a low density in mouse lymphoid tissues, resulting in insufficient cell-to-cell interaction of the immune cells as discussed by Sandhu et al. [22].

To overcome these problems, we implanted human CD34+CB cells into NOG or NOD-SCID mice to induce the development of human immune cells that adapt to the murine environment. In these reconstituted mice, considerable numbers of T and B cells develop and accumulate in the murine lymphoid tissues [5, 25]. To avoid GVHD, we are careful to purify CD34+CB cells before their implantation by double-positive selection using MACS beads and FACS [8], and have succeeded in efficient reconstitution of human T and B cells by CD34+ cells derived from CB, BM, and mobilized PBL.

2.2 *Antigen-Specific IgM Antibody is Dominant in Immunized CB-NOG Mice*

To determine whether antigen-specific antibody is produced in CB-NOG mice after immunization, CD34+CB cells purified by the double selection process were implanted into irradiated NOG mice. After development of human T and B cells was confirmed by marking the expression of CD3 and CD19/IgM, respectively, in peripheral blood, CB-NOG mice were immunized six times with DNP-KLH every 2 weeks. Figure 1 shows representative results of the raised antibody in immunized CB-NOG mice. Total immunoglobulin levels of IgM and IgG in the serum were equally increased after immunization. However, the serum level of antigen-specific IgG (anti-DNP-KLH) was detected but was extremely low, if detectable, in comparison with the IgM. Moreover, only two of nine mice had detectable IgG, while specific IgM was found in all experimental mice. In addition to hapten-carrier antigen, similar results were obtained when CB-NOG mice were immunized with other antigens such as OVA, OVA peptide, and superantigen (data not shown). Ishikawa et al. [6] reported similar results showing that specific IgG against OVA is detectable in NOG mice if the newborn mice are transplanted with human stem cells, although the antibody levels are very low . These results indicate that the CB-NOG mouse environment permits human B cell development to spontaneously produce IgG as well as IgM but may not be able to induce antigen-specific IgG antibody, which will be discussed later.

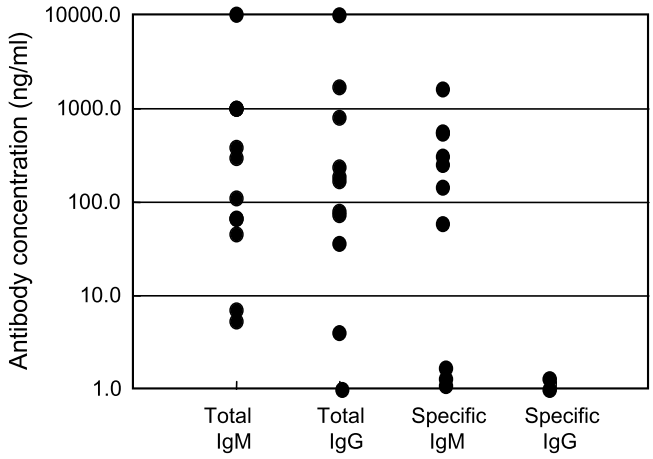

Fig. 1 Antibody level of total and antigen-specific IgM and IgG in immunized CB-NOG mice. NOG mice (n=11) were immunized with DNP-KLH emulsified with alum 6 times biweekly after implantation of human CD34+CB cells. After the last immunization at the 10th week, the concentration of anti-DNP-KLH in the mouse serum was measured by ELISA

2.3 Human CD34+Cells Preferentially Develop into CD5+B Cells in the CB-NOG Spleen

We have demonstrated that more than 50% of CD19+ cells in the CB-NOG mouse are CD5 positive but CD3 negative. This CD5 predominance did not change when CB, BM, or PBL was used as the CD34+ cell source [13]. These CD5+ B cells also expressed IgM, IgD, and CD20 in addition to CD19, indicating that human CD34+ cells develop into mature type CD5+B cells in the NOG environment. CD5+B cells belong to a different B cell subpopulation, termed B1 cells, that are distinguished from ordinary B cells, termed B2 cells [9, 10, 18]. At the same time, CD5+B cells are known to produce mainly IgM but are less likely to produce antigen-specific IgG. Such characteristics of CD5+B cells may provide a clue to explain why antigen-specific IgG human antibody is not raised very much in immunized CB-NOG mice.

Why do human CD5+B cells develop with high efficiency in NOG mice? Under physiological conditions, the proportion of CD5+B cells is known to be low in PBL and spleen, and CD5+B cells are predominantly located in the peripheral cavities of mice [25]. Moreover, CD5+B cells are rare in human tissues. Matsumura et al. [13] found that the proportion of CD5+B cells increased in the NOG spleen with time after transplantation but was low without an increase in BM. In fact, CD5-IgM-CD19+ cells became CD5+B cells in cocultures with nonreconstituted NOG spleen cells. In light of these findings, it is possible to speculate that the murine spleen environment may possess the potential to force human CD34+ cells to become the CD5+B cell lineage or may induce CD5+B cell proliferation and/or accumulation. At present, we do not have any suitable evidence to support either possibility, but our findings should provide tools for studying the development of human CD5+B cells, which is still controversial.

3 Limited Function of Human T Cells Developed in the Murine Thymus

3.1 IL-2 Production is Defective in T Cells Activated with Immunized Antigen in CB-NOG Mice

Since T cell help is required for a class switch of immunoglobulin from IgM to IgG after immunization, one may ask the question of whether human T cells developed in the CB-NOG mouse possess helper function in NOG mice in the same way as they develop physiologically in the human thymic environment. Past reports including ours demonstrated that human CD34+CB cells develop into CD4 and CD8 single-positive (CD4+ and CD8+) cells through CD4−8− double-negative (DN) cells and CD4+8+ double-positive (DP) cells in the murine thymic environment in vitro [21, 28] and in vivo [8, 21]. Single-positive thymocytes expressed mature type

T cell markers such as CD1a low and TCR high, and possessed the potential to produce cytokines after stimulation with the mitogen PMA/ionomycin [21]. In CB-NOG peripheral lymphoid tissues, human T cells developed from CD34+CB cells also express mature T cell markers [8].

In this study, we examined the cytokine-producing ability of human T cells after they have left the thymus in CB-NOG mice. When CD3high+ cells isolated from the CB-NOG spleen were stimulated with anti-CD3 or PMA/ionomycin in vitro, they expressed high levels of CD25, CD69, and CD154 (CD40L) and produced IL-2 in the culture supernatants (Fig. 2a, b), indicating that human T cells as well as mature thymocytes are activated to produce cytokine by TCR-mediated signaling. However, when CD3high+ T cells were isolated from the spleen of a CB-NOG mouse immunized with DNP-KLH and were cultured with nonreconstituted NOG spleen cells in the presence of the same antigen, their IL-2 production ability (Fig. 2c) and cell proliferation ability (data not shown) were extremely low in comparison with those

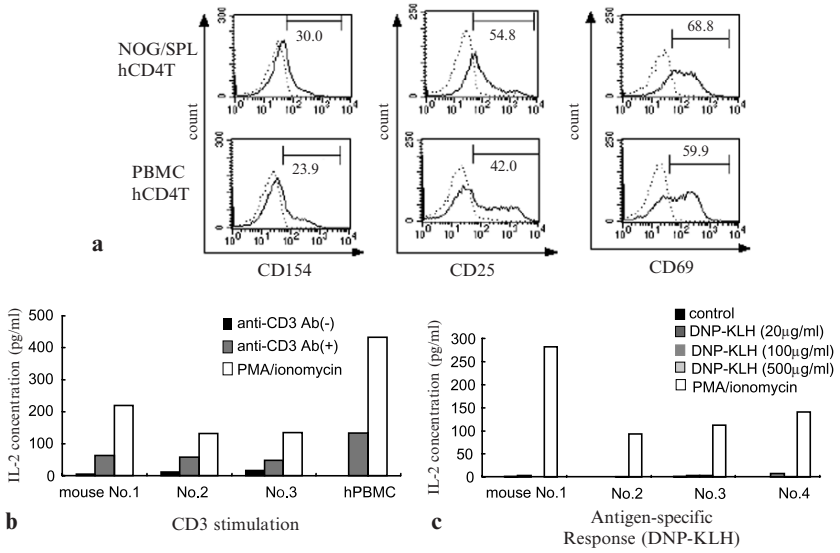

Fig. 2 Human T cells developed in CB-NOG mice are not activated by conventional antigens but activated by TCR-crosslinking. **a, b** T cells collected from CB-NOG spleen without stimulation (NOG/SPL hCD4T) or from PBL (PBMC hCD4T) of healthy volunteers were stimulated with anti-human CD3 antibody or PMA/Ionomycin. The in vitro stimulated T cells were subjected to flow cytometric analysis to determine the expression of activated antigens CD154 (16-h culture), CD25 and CD69 (48-h culture) (**a**). The concentration of human IL-2 in the culture supernatants was measured by ELISA. *Closed squares*, cells without anti-CD3 treatment. *Gray squares*, cells with anti-CD3 treatment. *Open squares*, cells treated with PMA/ionomycin (**b**). **c** CB-NOG mice were immunized with DNP-KLH emulsified with alum biweekly. One week after the 3rd booster, CD4 T cells collected by a magnetic bead system were cocultured with mitomycin C-treated non-immunized spleen cells without T cells in the presence of serially diluted DNP-KLH (20-500 μg/ml) for 48-h. The IL-2 concentration of the cultured supernatants was measured by ELISA. *Control cells*, cells cultured without stimulation

of the stimulated T cells with PMA/ionomycin or superantigen TSST-1. Why do T cells in the immunized mouse not respond properly to a given antigen such as DNP-KLH, despite the fact that human T cells appeared in the CB-NOG spleen and they can be induced to produce cytokine through the TCR signaling pathway in vitro?

3.2 Human T Cells Are Positively Selected by Murine MHC in the NOG Thymus

One possible interpretation of the reduced response to T cells by the immunized mice is that human T cells developed in the NOG mouse do not appropriately recognize antigen peptide presented by the human MHC on human antigen-presenting cells (APC) because human T cells are positively selected by the murine MHC in the NOG thymus. To confirm this assumption, we examined whether or not human T cells substantially develop in the NOG thymus under the restriction of the murine MHC. For this purpose, we used a reaggregate thymic organ culture (RTOC) system [2, 23] in which hematopoietic stem cells are cocultured with thymic stromal cells by generating a three-dimensional structure. In RTOC, human CD34+CB cells were cocultured with thymic stromal cells obtained from the fetal thymus of MHC class II (I-Ab) knockout (KO) mice or wild-type (WT) C57BL/6 mice to generate human/murine hybrid culture clusters. The clusters were then engrafted beneath the NOG kidney capsule, and 8 weeks later cells in the clusters were subjected to flow cytometric analysis. The developing cells in RTOC showed that the proportions and total cell numbers of human CD4+cells were reduced in KO mouse-derived hybrid clusters in comparison with WT-derived clusters while CD8+ cells were intact (Fig. 3).

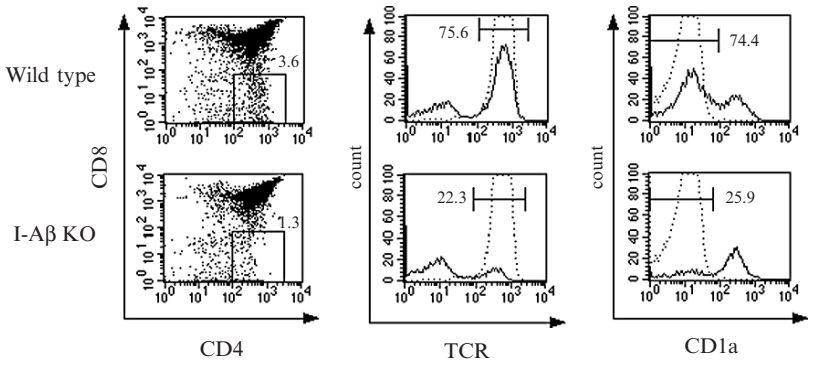

Fig. 3 Human T cells are positively selected by the murine MHC. Fetal thymic stromal cells obtained from C57BL/6 or I-Ab KO mice were cocultured with human CD34+CB cells to generate human/murine hybrid clusters. One week after reaggregation culture, the hybrid cluster was transplanted beneath the kidney capsule of NOG mice. Eight or 10 weeks after transplantation, lymphoid cells collected from the reaggregated clusters were stained with antibodies against human CD45, CD4, CD8, CD1a and TCR for flow cytometric analysis

Moreover, these CD4+ cells obtained from KO mouse-derived hybrid clusters expressed high CD1a+ and low CD3 (immature T cell markers) at relatively high rates. These results indicate that the majority of human T cells are positively selected to reach maturity under the restriction of the murine MHC in the NOG thymus.

The CB-NOG spleen may contain large numbers of human-derived hematopoietic cells but less murine cells because NOG mice received irradiation before CB cell implantation. Moreover, no B cell is present in NOG mice. Thus, human T cells developed under murine MHC restrictions have little chance to encounter murine APC including B cells for accomplishing cognate interaction for activation as memory cells. At the same time, these human T cells can not interact with human B cells and DC because they express human MHC. Collectively, it is strongly suggested that human T cells in the immunized CB-NOG mice respond poorly to in vitro restimulation with the immunized antigen because most of their TCR repertoire is restricted to murine MHC.

4 Antigen-Specific IgG Antibody in NOG Mice with Human Lymph Node Engraftment

When human T cells develop in the context of murine MHC restriction in CB-NOG mouse as mentioned above, they are unlikely to show suitable interaction with B cells expressing human MHC in CB-NOG mice, resulting in the failure of memory T cell development, which is responsible for the class switch from IgM to IgG. The ideal method to overcome the impediment of MHC restriction is to establish a system in which human T cells develop selectively in the murine thymic environment expressing human MHC. If the MHC gene of the NOG mice is replaced with the human MHC gene, T cell repertoires will be positively selected under human MHC restriction even in the NOG thymus. In such a mutant mouse, developed human T cells can be stimulated with given antigens that are presented by human MHC class II, resulting in induction of IgG antibody production in B cells through matched TCR-MHC interaction. This trial is now in progress.

Another system in which the human immune system can function under the same MHC restriction in laboratory animals is the engraftment of human lymphoid tissues in the immunodeficient mouse. In the initial studies, human embryonic thymus was implanted with human hematopoietic stem cells or embryonic hematolymphoid tissues in SCID mice, typical immunodeficient animals [14, 17]. This experimental system may enable T cells to interact with APC and with B cells via the human MHC in NOG mice, but it is not practical because human fetal tissues cannot be obtained because of ethical considerations. However, if human lymphoid tissues or organs are obtained in a surgical operation with informed consent and are implanted in NOG mice, the ethical problem may be solved. The advantages of the NOG mouse include the fact that it is an excellent recipient of human tissues of high frequencies and that human immune cells can remain in engrafted tissues for relatively long periods, so that organ implantation in NOG mice can overcome the

disadvantage that may occur with implantation of human PBL as described previously.

Recently, we generated NOG mice with engraftment of adult human lymph nodes (LN) to promote human antigen-specific IgG production. In these experiments, one-quarter of the human LN, which were obtained with informed consent from patients with breast cancer, were implanted on the surface of the NOG spleen. These mice (abbreviated as LN-NOG) were immunized with DNP-KLH according to the protocol shown in Fig. 4. After immunization, the antibody level in the serum was examined every 2 weeks. Two out of 12 LN-NOG mice did not show any trace of engrafted LN tissues when they were sacrificed at the 7th week, and no T and B cells were detected in the spleen. In the remaining 10 LN-NOG mice bearing the engrafted LN, the antigen-specific antibody level was significant in 9 mice, with both IgM and IgG found in 7 mice and IgG alone and IgM alone in 1 mouse each (Fig. 5). One in 10 mice with engrafted LN died within 4 weeks but showed a slight increase of antigen-specific antibody in the 3rd week. In the LN-NOG spleen, numerous T and B cells were found and were analyzed by immunohistochemistry (Fig. 6) and flow cytometry (data not shown). In contrast, human-derived lymphoid cells were almost undetectable in murine LN of the inguinal and brachial regions, although decreased numbers of human T and B cells remained in the implanted human LN. These findings indicate that human immune cells of engrafted LN migrate into the contiguous spleen, where they proliferate and are activated efficiently

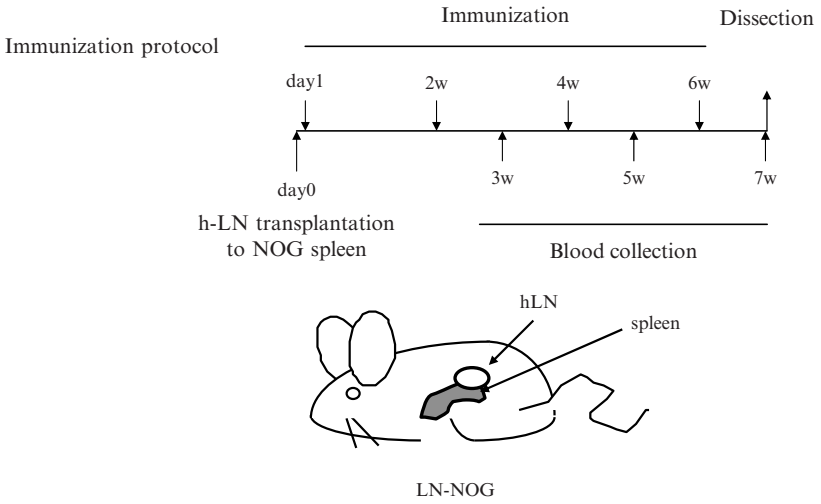

Fig. 4 Experimental protocol of lymph node engraftment and immunization in NOG mice. Nine-week-old NOG mice (n=13) were irradiated and transplanted with one-quarter of human lymph nodes on the spleen (LN-NOG). One day after transplantation, immunization with DNP-KLH was started biweekly. One week after each immunization, sera were collected for titration of antibodies. One week after the 3rd booster, LN-NOG mice were sacrificed for further analysis

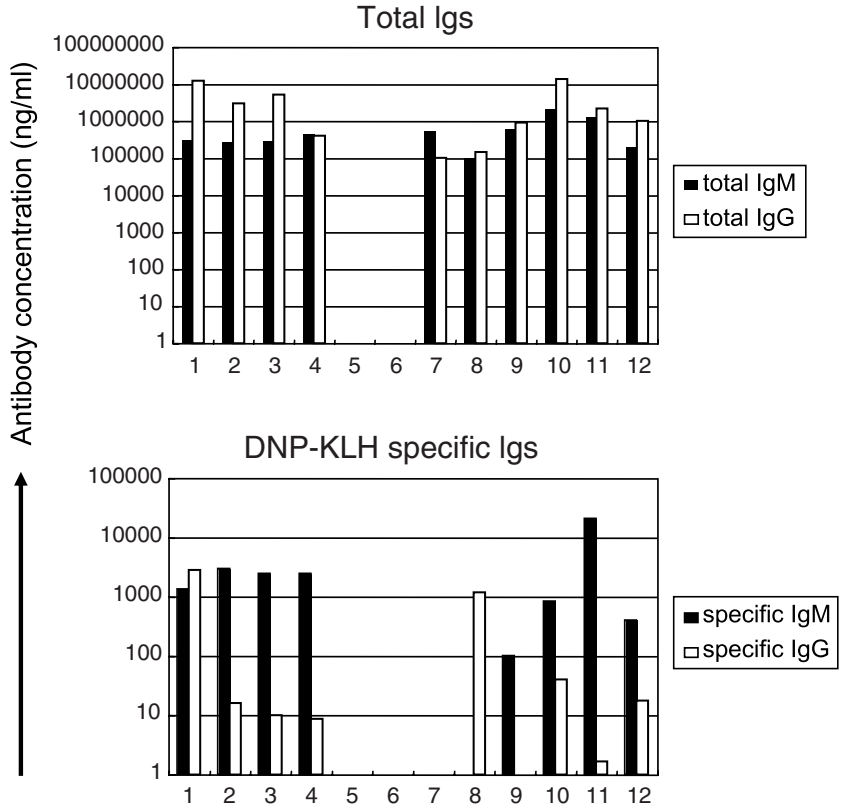

Fig. 5 Antibody production of antigen-specific IgG in LN-NOG mice immunized with DNP-KLH. NOG mice were immunized with DNP-KLH as described in Figs. 1 and 3. The serum concentration of antibodies (total, antigen specific IgG and IgM) was measured 3 times biweekly by ELISA. Each *bar* of the antibody concentration shows the highest level during immunization. *Closed bars*, human IgM. *Open bars*, human IgG. *Upper panel*, total human IgM and IgG. *Lower panel*, DNP-KLH-specific IgM and IgG. The *arabic numerals* on the *x-axis* represent the individual mouse numbers

with given antigens injected intraperitoneally. Our results are consistent with a previous report in which antigen-specific antibody was detected in SCID mice with engraftment of liver, thymus, skin fragments, and LN of the human embryo [3]. Taken together, it was suggested that if mature human cell components involved in immune reactions accumulate in a limited region for mutual interaction, suitable IgG antibody production for the given antigen can be induced in the mouse environment. In this context, LN-NOG mice are convenient for obtaining antigen-specific antibodies derived from human B cells, which would be risk-free therapeutic reagents.

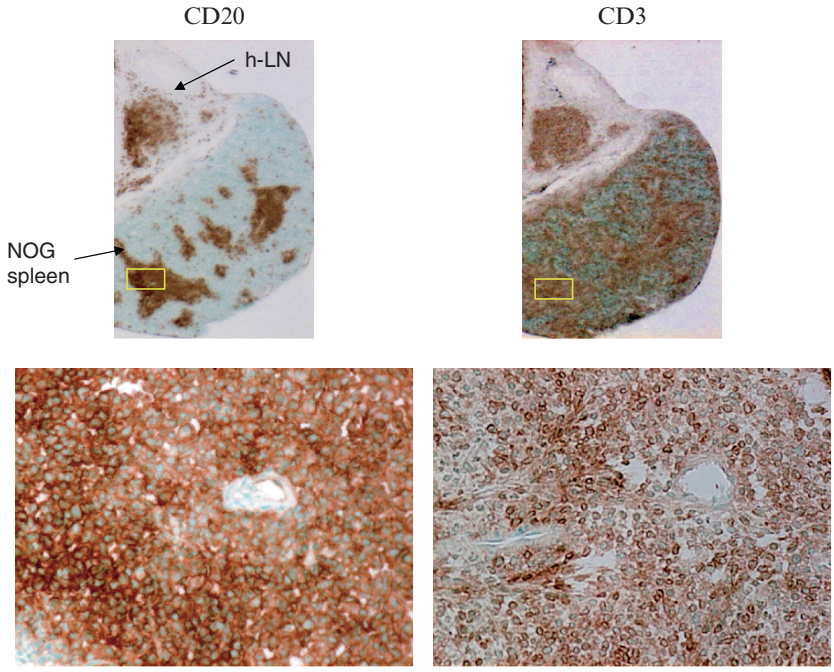

Fig. 6 Human T cells are detectable in NOG spleen and engrafted human lymph node. LN-NOG mouse tissues were subjected to immunohistochemical analysis by staining with anti-human CD20 or anti-human CD3 mAb. The *lower panels* show the magnification of the *boxed areas* of the *upper panels*

5 Conclusion

In NOG mice reconstituted with human CD34+CB cells, mature type human T and B cells developed well but antigen-specific IgG antibody was produced at an extremely low frequency and a low level, if at all, when these mice were immunized with an antigen. Human T cells developed in the mouse environment possess the potential to produce T cell-specific cytokines through TCR-mediated signaling, but it seems likely that they cannot efficiently recognize antigens presented by human APC and B cells because of human T cells positively selected by the murine MHC during the intrathymic development process in the mouse. As a result, memory T cells do not develop to help in class switching of immunoglobulin from IgM to IgG in B cells. Therefore, to obtain antigen-specific IgG antibody derived from human B cells, it is essential to overcome the restriction of the murine MHC in T cell development.

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