U1B3.3 monoclonal antibody recognizes a precursor of NK1.1⁺ cytotoxic T cells generated by interleukin-2

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Abstract: U1B3.3 monoclonal antibody (mAb) was established by immunizing a rat with the tMK-2U lymphoma cell line, derived from athymic nude mice. U1B3.3 mAb recognizes some T cell populations, B cells and natural killer (NK) cells. U1B3.3⁺ T cells express CD3/T cell receptor (TCR) complex at a unique intensity: between that of TCR-intermediate cells (TCR^{int} cells) and TCR^{bright} cells. TCR^{int} cells strongly express the interleukin-2 receptor β chain (IL-2R β), but almost all U1B3.3⁺ T cells express high or low levels of IL-2R β . When purified U1B3.3⁺ NK1.1⁻ T cells were cultured with IL-2, approximately 14% of the cells acquired expression of NK1.1 molecules, which are expressed on NK cells and natural killer T (NKT) cells. Furthermore, U1B3.3⁺ NK1.1⁻ T cells killed tumor cells after culture with IL-2. These results indicate that U1B3.3 mAb recognizes a precursor of NK1.1⁺ cytotoxic T cells generated by IL-2.

Key words: U1B3.3 mAb (monoclonal antibody); NKT (natural killer T) cell; TCR intermediate (TCR^{int}) cell; IL-2 (interleukin-2); IL-2R β (receptor β -chain).

Introduction. The nonclassical major histocompatibility complex (MHC) class I molecule CD1d presents glycolipid or lipid antigens. CD1d-restricted T cells designated as natural killer T (NKT) cells co-express the NK1.1 molecule, a marker found on natural killer (NK) cells.¹⁾⁻³⁾ NKT cells have a skewed T cell receptor (TCR) repertoire, with most expressing an invariant TCR- α chain (V α 14) and TCR- β chain (V β 8.2, V β 2 or V β 7), and comprise CD4⁺ and CD4⁻CD8⁻ cell populations. TCR expression of NKT cells is intermediate between that of mature T cells and thymic immature T cells. NKT cells are therefore also called intermediate TCR cells (TCR^{int} cells).^{4),5)} CD1d-restricted NKT cells can produce large amounts of interferon (IFN)- γ and interleukin (IL)-4, and regulate T helper 1 and T helper 2 immune responses. They can prevent autoimmune disease, and are strong antitumor effectors.⁶⁾⁻⁸⁾

Recent studies have demonstrated that NK1.1 molecules can be expressed by MHC class I- and II-restricted T cells after cytokine stimulation or viral infection.⁹⁾⁻¹²⁾ These CD1d-unrestricted NK1.1⁺ T cells can produce IFN γ and show cytotoxic activity after IL-2-stimulation.¹³⁾⁻¹⁵⁾ They have been found in peripheral

immune organs and the large intestine,^{16),17)} and in aged athymic nude mice.¹⁸⁾ Previously, we showed that a subpopulation of NK1.1⁻ IL-2 receptor β -chain (IL-2R β)⁺ T cells could express NK1.1 molecules and exhibit cytotoxicity against tumor cells after IL-2 stimulation *in vitro*.⁹⁾ It has been reported that these IL-2-induced NK1.1⁺ T cells are CD1d-unrestricted.¹⁰⁾

In the present study, we established the monoclonal antibody (mAb) U1B3.3. This mAb was raised against the NKT-cell-like lymphoma cell line tMK-2U, which unexpectedly developed in BALB/c nude mice after implantation of human inflammatory breast cancer.¹⁹⁾ U1B3.3 recognized a unique T cell population that expresses TCR at an intensity between that of TCR^{int} cells and TCR^{bright} cells, and recognized IL-2R β ⁺ cells but not IL-2R β ⁻ T cells. Furthermore, we demonstrated that a subpopulation of U1B3.3⁺ T cells acquired expression of NK1.1 molecules and killed tumor cells after IL-2 stimulation.

Materials and methods. *Animals.* Male Fischer rats and female C57BL/6N mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan). All mice were used at 10 to 20 weeks of age, and were maintained in our facilities.

Antibodies. The following antibodies were used in

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this study: FITC- or R-PE-conjugated goat F(ab')₂ anti-rat IgG (Caltag, San Francisco, CA, U. S. A.), FITC-conjugated anti-rat IgG 1(R3-34; PharMingen, San Diego, CA, U. S. A.), PE-conjugated anti-IL-2R β (TM- β 1; PharMingen), PE-conjugated anti-NK1.1 (PK136; PharMingen), biotin-conjugated anti-CD3 (145-2C11; PharMingen), and biotin-conjugated anti-TCR- β (H57-597; PharMingen).

Hybridoma production and screening.

Hybridoma production is described in detail elsewhere.²⁰⁾ Briefly, a rat was immunized with the tMK2-U lymphoma cell line. Lymph node cells from the rat were fused with a mouse myeloma (P3-X63-Ag8). Hybridomas selected by immunofluorescence staining.

Cell preparation and cell culture. Preparation of hepatic mononuclear cells (MNCs) is described elsewhere.⁹⁾ Cells were cultured in RPMI-1640 medium supplemented with 8% FCS, 5×10^{-5} M 2-ME, 2 mM L-glutamine, 1 mM sodium pyruvate, 3.6% glucose, 100 U/ml penicillin, 100 μ g/ml streptomycin and 1000 U/ml recombinant human IL-2 (kindly provided by Takeda Chemical Ind., Ltd., Osaka, Japan).

Immunofluorescence analysis and cell sorting.

Surface phenotype of cells was determined using two- or three-color flow cytometry, as described elsewhere.^{9),20)} Briefly, 5×10^5 cells were incubated with culture supernatant of U1B3.3 hybridoma and secondary FITC-conjugated goat (Fab')₂ anti-rat IgG. They were then incubated with mouse serum, and then incubated with PE- and biotin-conjugated antibody with PerCP-conjugated streptavidin (Becton Dickinson, San Jose, CA, U. S. A.), Cy-Chrome-conjugated streptavidin (PharMingen) or FITC-avidin (Vector Laboratories, Burlingame, CA, U. S. A.). Stained cells were analyzed with a flow cytometer (FACS Calibur, Becton Dickinson). To obtain hepatic U1B3.3⁺NK1.1⁻ T cells and NK1.1⁺ T cells, cell sorting was performed using FACS Vantage (Becton Dickinson), as described elsewhere.⁹⁾

Cell-mediated cytotoxicity. Effector cells were tested for cytotoxicity against YAC-1 cells and concanavalin A-stimulated splenic blast cells, using a ⁵¹Cr release assay, as described elsewhere.⁹⁾

Results and discussion. To generate mAb specific for NKT cells with an intermediate level of TCR, we immunized a rat with a tMK-2U cell line that was similar to TCR^{int} cells derived from athymic BALB/c *nu/nu* mice.^{19),20)} We previously reported obtaining U5A2-13 mAb, which recognizes a phenotypically and functionally similar population of TCR^{int} cells or NKT cells.^{21),22)} During the experiment in which we obtained U5A2-13

Fig. 1. Establishment of U1B3.3 mAb. Hepatic MNCs of C57BL/6 mice were analyzed with two-color flow cytometry after staining with FITC-conjugated CD3 and one of the following: (A) U1B3.3 mAb plus R-PE-conjugated anti-rat IgG. (B) U5A2-13 mAb plus R-PE-conjugated anti-rat IgG. (C) PE-conjugated anti-IL-2R β .

mAb, we also obtained U1B3.3 mAb (rat IgG1 isotype), which recognizes a unique T cell population. U5A2-13 mAb recognized TCR^{int} cells (Fig. 1(B)), whereas U1B3.3 mAb recognized T cells expressing TCR/CD3 at an intensity between that of TCR^{int} cells and TCR^{bright} cells (Fig. 1(A)). Previously, it was reported that TCR^{int} cells can be easily distinguished from TCR^{bright} cells

Fig. 2. Percentage of U1B3.3⁺ or U5A2-13⁺ T cells among IL-2R β^{bright} T cells, IL-2R β^{low} T cells and IL-2R β^- T cells. Hepatic MNCs of C57BL/6 mice were analyzed with three-color flow cytometry after staining with U1B3.3 or U5A2-13 mAb plus FITC-conjugated goat F(ab')₂ anti-rat IgG, biotin-conjugated CD3 plus PerCP-conjugated streptavidin and PE-conjugated anti-IL-2R β . The cells were gated into 3 subsets: IL-2R β^{bright} T cells, IL-2R β^{low} T cells, or IL-2R β^- T cells, and U1B3.3 or U5A2-13 expression was analyzed.

since this population strongly express LFA-1, IL-2R β or CD44 molecules.^{5),23)} Our results indicate that 3 distinct levels of TCR intensity can be distinguished using U1B3.3 mAb. U1B3.3 mAb also recognized B cells, NK cells and a few NK1.1⁺ T cells (data not shown).

Hepatic T cells were classified into 3 distinct populations based on expression level of IL-2R β : IL-2R β^- , IL-2R β^{low} and IL-2R β^{bright} (Fig. 1(C)). Almost all U5A2-13⁺ T cells belonged to the IL-2R β^{bright} T cell population (Fig. 2(B)). Next, we examined the expression level of IL-2R β on U1B3.3⁺ T cells. As shown in Fig. 2(B), 12.6% of IL-2R β^{bright} T cells, 47.8% of IL-2R β^{low} T cells and 6.2% of IL-2R β^- T cells expressed U1B3.3 antigen. These results indicate that the majority of U1B3.3⁺ T cells express IL-2R β and are therefore responsive to IL-2.

Previously, we reported that IL-2R β^+ NK1.1⁻ T cells could acquire expression of NK1.1 molecules after incubation with IL-2.⁹⁾ To determine whether U1B3.3⁺

NK1.1⁻ T cells can be made to express NK1.1 molecules using IL-2 stimulation, we isolated both U1B3.3⁺ NK1.1⁻ TCR- β^+ cells and NK1.1⁺ TCR- β^+ cells from hepatic MNCs using a cell sorter. As shown in Fig. 3, after incubation with IL-2, NK1.1 molecules were expressed on 14% of U1B3.3⁺ NK1.1⁻ TCR- β^+ cells (Fig. 3(B)), and 88% of sorted NK1.1⁺ T cells retained expression of NK1.1 (Fig. 3(C)). Similar results were obtained using anti-CD3 instead of anti-TCR- β mAb (data not shown). This suggests that U1B3.3 mAb recognizes a precursor of IL-2-induced NK1.1⁺ T cells. These IL-2-induced NK1.1⁺ T cells were distinct from CD1d-restricted NKT cells because they were also found in CD1d^{-/-} mice.¹⁰⁾ Several groups have reported finding that CD1d-unrestricted NKT cells are mainly CD8⁺, unlike CD1d-restricted NKT cells, and have reported finding CD1d-unrestricted NKT cells in peripheral immune organs.^{16),17)} Recent studies have shown that some

Fig. 3. NK1.1⁻ U1B3.3⁺ T cells acquire expression of NK1.1. Hepatic MNCs of C57BL/6 mice were stained with U1B3.3 mAb plus FITC-conjugated anti-rat IgG1 mAb, PE-conjugated-NK1.1 and biotin-conjugated CD3 plus Cy-Chrome-conjugated streptavidin. Cells were then sorted using FACS Vantage. Purified U1B3.3⁺ NK1.1⁻ T cells (B), U1B3.3⁻ NK1.1⁺ T cells (C) and unseparated cells (A) were cultured with IL-2 (1000 U/ml) for 7 days, and phenotype was then examined. The cells were stained with biotin-conjugated CD3 plus FITC-conjugated streptavidin and PE-conjugated anti-NK1.1, and were analyzed using FACSCaliber.

Table I. Cytotoxic activity of U1B3.3⁺ NK1.1⁻ T cells and NK1.1⁺ T cells after IL-2 stimulation

Target cells	Effector cells ^{a)}	Percent specific lysis		
		10:1	3.3:1	1.1:1 ^{b)}
YAC-1	U1B3.3 ⁺ NK1.1 ⁻ T cells	16.4	6.1	2.1
	NK1.1 ⁺ T cells	40.6	26.3	15.1
Con A blast ^{c)}	U1B3.3 ⁺ NK1.1 ⁻ T cells	2.8	-0.8	1.9
	NK1.1 ⁺ T cells	0.2	-2.8	-0.6

^{a)} Sorted U1B3.3⁺ NK1.1⁻ T cells and NK1.1⁺ T cells were cultured for 7 days in medium containing IL-2 (1000 U/ml). After culture, the cells were examined for cytotoxic activity against YAC-1 cells and syngeneic concanavalin A (Con A) blast cells, using a 4 h. ⁵¹Cr-release assay. The data presented are percentage of specific lysis. ^{b)} Effector : Target (E:T) ratios. ^{c)} Spleen cells of syngeneic C57BL/6 mice were cultured with Con A for 2 days.

influenza-specific CD8⁺ T cells express NK1.1 molecules,¹¹⁾ and that CD8⁺ NKT cells are expanded in tumor bearing mice, aged athymic mice and thymectomized bone marrow chimera mice.^{14),18),23)} Furthermore, Matsuda *et al.*²⁴⁾ and Benlagha *et al.*²⁵⁾ produced CD1d-glycolipid tetramers and clearly identified CD1d-restricted NKT cells among the various kinds of NK1.1⁺ T cells. We also produced CD1d tetramers and examined whether IL-2-induced NK1.1⁺ T cells were CD1d-restricted. We found few CD1d-restricted NK1.1⁺ T cells after incubation of hepatic

MNCs with IL-2 (Kanai and Ikarashi *et al.*, paper in preparation). Together, these findings and the present results indicate that there is a distinct T cell population composed of classical CD1d-restricted NKT cells that express NK1.1 molecules when activated *in vivo* or *in vitro*.

Previously, we reported that NK1.1⁻ IL-2R β ⁺ T cells exerted cytotoxic activity after IL-2 stimulation.⁹⁾ Furthermore, it has been reported that NK1.1⁺ T cell also exhibit strong cytotoxicity after IL-2 stimulation.²⁷⁾ We examined whether U1B3.3⁺ NK1.1⁻ TCR- β ⁺ cells could also exert NK cell-like cytotoxic activity after IL-2 stimulation. U1B3.3⁺ NK1.1⁻ TCR- β ⁺ cells and NK1.1⁺ TCR- β ⁺ cells were cultured in medium containing IL-2 for 7 days, and cytotoxic assays were then performed. As shown in Table I, after IL-2 stimulation, both U1B3.3⁺ NK1.1⁻ TCR- β ⁺ cells and NK1.1⁺ T cells showed cytotoxic activity against NK cell-sensitive YAC-1 cells but not against syngeneic concanavalin A-stimulated blast cells. Recently, it was reported that activated CD8⁺ NKT cells exhibit cytotoxicity against multiple tumor cell types, and that this activity is independent of CD1d and MHC class I molecules.¹⁴⁾ This activity may involve NK1.1 or another NK cell receptor such as NKG2D.²⁸⁾ In addition, CD8⁺ NKT cells can produce levels of IFN γ similar to those of CD1d-restricted NKT cells. These CD1d-unrestricted NKT cells may function as part of innate immu-

nity.

In livers of athymic nude mice, some T cells extrathymically develop and gradually expand with aging.¹⁸⁾ These extrathymically developed T cells express intermediate levels of TCR and IL-2R β . Recently, it has been shown that some CD8⁺ IL-2R β ⁺ T cells, but not IL-2R β ⁻ T cells, develop in extrathymic organs such as the liver.^{4),15)} The fact that U1B3.3 mAb recognized hepatic IL-2R β ⁺ T cells and was established by immunizing a rat with tMK-2U lymphoma cells which are like TCR^{int} cells derived from athymic BALB/c *nu/nu* mice suggests that U1B3.3 mAb recognizes some extrathymic T cell populations and is useful for analysis of extrathymic T cells. The protein structure and cDNA sequence of U1B3.3 antigen is therefore an interesting subject for future study.

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