Generation and Characterization of Two Novel Monoclonal Antibodies Produced Against Human TLT-2 Molecule

Jun-Chi Xu,^{1*} Fei Gao,^{2*} Feng-Qing Fu,² Yong-Jin Chen,² Ping Xu,¹ Bin Zhou,² and Xue-Guang Zhang²

Trem-like transcript 2 (TLT-2), one of the TREM family members, which is expressed on B cells, T cells, and macrophages, plays a critical role in immune response mechanism. In this study, two novel mouse anti-human TLT-2 monoclonal antibodies (MAbs) were prepared using hybridoma technology and their immunological characteristics were determined. The results showed that the two MAbs (clones 10F5 and 8C10) were both IgG1 (κ) and bound specifically to human TLT-2. Furthermore, 10F5 and 8C10 seemed to recognize a different site (epitope) of TLT-2 by competition assay. MAb 10F5 was proven in Western blot analysis to specifically bind to denatured TLT-2 protein while both MAbs were proven in dot blot analyses and immunofluorescence to specifically bind to natural TLT-2 protein. In addition, crosslinking of TLT-2 with MAb 8C10 markedly blocked TLT-2 positive signal and T cell proliferation. Taken together, these two monoclonal antibodies might be of great value as tools for further exploration of the expression and function of TLT-2.

Introduction

THE INNATE IMMUNE SYSTEM IS AN ANCIENT FORM of host defense that relies upon a wide range of pattern recognition receptors,⁽¹⁻⁴⁾ such as toll-like receptors (TLR),^(5,6) G-protein coupled receptors, mannose receptors, and Fc receptors, which are expressed on neutrophils, monocytes-macrophages, NK cells, and other immune cells. These receptors can recognize and bind pathogens, then mediate effector cells playing an immune function. In addition to these receptors, effector immune cells express other pattern recognition receptors, which often include stimulate and inhibitory receptors.⁽⁷⁻¹³⁾ Triggering receptors expressed by myeloid cells (TREM) are an example of one of these kinds of receptor.⁽¹⁴⁾

Trem-like transcript 2 (TLT-2) gene is one of the conserved TREM family members, which is expressed on B cells,⁽¹⁵⁾ T cells, macrophages, and neutrophils.⁽¹⁶⁾ The mouse TLT-2 is suggested to play an important role in the immune system, for it is not only highly expressed in inflammation, but also promotes the proliferation of T cells and the production of cytokines. However, the biological function of TLT-2 remains unclear and is still controversial. Masaaki and colleagues have shown that TLT-2 interacts with B7-H3 expressed on accessory cells and that this promotes enhanced T cell activation.^(17,23,24) But Leitner and colleagues found that there is no evidence for B7-H3 and TLT-2 interaction.⁽¹⁸⁾ At the same time, the biological function of the B7H3 is also in dispute.⁽¹⁷⁻²⁴⁾

Although several studies have reported on the generation of some monoclonal antibodies (MAbs) against human TLT-2,⁽²³⁾ up until now only two of these were found to reveal agonistic or antagonistic functions and most of these antibodies can only be applied to flow cytometry, immunohistochemistry, or Western blot analysis.⁽²⁴⁾ In this study we successfully generated two mouse anti-human TLT-2 monoclonal antibodies (MAbs 10F5 and 8C10). When the biological character of these two MAbs was studied, we proved that the epitope recognized by 10F5 and 8C10 is different by competition assay. TLT-2 was elucidated and widely and distinctively expressed on many tumor cell lines. Flow cytometric analysis showed that TLT-2 is highly expressed on monocytes and B cells and weakly expressed on T cells. The significance of TLT-2 expression on tumor cells and the underlying mechanisms need further investigation. Therefore, specific MAbs against the TLT-2 molecule are valuable tools for modulation of TLT-2 signal pathway. This not only provides a new perspective for a thorough understanding of the delicate regulation of the immune system, but also plays a potential role in clinical application.

Materials and Methods

Animals, cell preparation, reagents, and antibodies

Six-week-old BALB/c mice were purchased from the Department of Experimental Animal, Shanghai Institute of Biological Products, Ministry of Health of China. Murine

¹The Fifth People's Hospital of Suzhou, China.

²Medical Biotechnology Institute of Soochow University, Suzhou, China.

*These authors contributed equally to this work.

FIG. 1. Successfully established L929/TLT-2 transfectant cell lines. (A) L929/TLT-2 transfectants stably expressing GFP were selected and identified by flow cytometry. (B) Analysis of gene expression by RT-PCR. Lane 1, L929/TLT-2 GAPDH; lane 2, L929/TLT-2 full length of TLT-2; lane 3, marker; lane 4, L929/mock GAPDH; lane 5, L929/mock TLT-2 full length of TLT-2. (C) Analysis of protein expression by Western blot analysis. Lane 1, Doudi + AF3259; lane 2, L929/TLT-2 + AF3259; lane 3, L929/mock + AF3259. Empty vector transfected L929 (L929/mock) were used as negative control. The figure is representative of three independent experiments. A shows that flow cytometry can identify GFP expression on the transfected cell line. RT-PCR can reveal TLT-2 expression at the gene level and Western blot can reveal its expression at the protein level.

myeloma cell line SP2/0, murine fibrous cell line L929, and all the human lines (Raji, Daudi, THP-1, M431, M231, Jurkat) were originally obtained from American Type Culture Collection (Manassas, VA). Human peripheral blood mononuclear cells (PBMCs) were prepared from healthy donors by Ficoll-Hypaque (Hengxin Chemistry Company, Shanghai, China) density-gradient centrifugation. Then the T cells were enriched from PBMCs by EasySep and the purity was >90%.

The above cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) or standard DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FCS (Hyclone, Logan, UT), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. Cells were cultured in a 5% CO₂, 37°C incubator. Cell counting kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). Goat anti-human TLT-2 polyclonal antibody (clone AF3259) was purchased from R&D Systems (Minneapolis, MN). PE-conjugated goat anti-mouse IgG, biotinylated mouse IgG1, agonistic mouse anti-human CD3 MAb (clone UCHT1), and mouse IgG1 (clone 679.1Mc7) were all purchased from Immunotech (Marseille, France). Functional mouse anti-human CD28 MAb was prepared in our laboratory.⁽²⁵⁾

Construction of transfectants

The complete cDNA encoding human TLT-2 was cloned from human tumor cell line Daudi by the reverse transcription polymerase chain reaction (RT-PCR) method with special primers (forward: 5'-CCTCTCGAGATGGCCCCAGCCTTCTGC-3'; reverse: 5'-TCGGATCCAGTAGACTTCCACATAGG-3') and was inserted into vector pIRES2-EGFP. The recombinant pIRES2-EGFP-TLT-2 was then transformed into chemically competent TOP 10 cells and selected by kanamycin resistance. The positive clones were then extracted. L929 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) reagent according to the manufacturer's protocol. Selected by G418 (Invitrogen) at 800 µg/mL, the TLT-2 transfected L929 cells (L929/TLT-2) were identified by RT-PCR, Western blot analysis, and flow cytometry. Empty vector-transfected L929 cell line (L929/mock) was also obtained. In addition, CD28 and B7-H3 transfectants were constructed by the same procedure.

Generation of anti-TLT-2 specific monoclonal antibodies

The MAbs were prepared using classical hybridoma technology. Six-week-old BALB/c mice were immunized a total of four times with injections of 1×10^7 mitomycin-treated L929/TLT-2 cells in 0.5 mL phosphate-buffered saline (PBS) per mouse at 21-day intervals. The first subcutaneous injection was accompanied by complete Freund's adjuvant (Sigma-

FIG. 2. Flow cytometric analysis of the MAb specificity. Reactivity of the two anti-TLT-2 MAbs with the transfectant L929/mock, L929/TLT-2, L929/CD28, and L929/B7-H3. Cells were stained with MAbs (10F5 and 8C10; grey histograms) or negative control mouse IgG1 (open histograms) at 4°C for 30 min, followed by goat anti-mouse IgG-phycoerythrin PE.

FIG. 3. Dot-blot and Western blot analysis. Only 10F5 MAb could recognize the denatured TLT-2 protein. Both MABs were proven in dot-blot to specifically bind to natural TLT-2 protein as compared with PBS and mouse IgG1 staining (negative control). **(B)** Analysis of gene expression by Western blot analysis. Lane 1, L929/TLT-2+AF3259; lane 2, L929/TLT-2+10F5; lane 3, L929/TLT-2+8C10; lane 4, L929/mock+AF3259; lane 5, L929/TLT-2+10F5; lane 6, L929/TLT-2+8C10.

Aldrich, St Louis, MO). Four days after the final boost injection, the splenocytes of immunized mice were fused with murine myeloma SP2/0 cells, according to the method described by Groth and Zhang. After hybridomas were selected by hypoxanthine-aminopterin-thymidine (HAT, Sigma-Aldrich), flow cytometry (Altra, Beckman Coulter, Miami, FL) was performed to screen positive clones. Hybridomas secreting mouse anti-human TLT-2 MABs were obtained by their strong reactivity with L929/TLT-2 cells, but not with L929/mock, L929/CD28, or L929/B7-H3 transfectants. The hybridomas were injected intraperitoneally into pristane-primed BALB/c mice for 8–10 days. The MABs were purified from ascites of BALB/c mouse using protein G-sepharose affinity columns (Pharmacia, Uppsala, Sweden).

Characterization of anti-TLT-2 MABs

For flow cytometric analysis, the transfected cells (1×10^6) mentioned above were incubated with MAB 10F5 or 8C10 for 30 min at 4°C and washed with PBS. For indirect staining, PE-conjugated goat anti-mouse antibody was added and incubated for another 30 min. After being washed with PBS, the stained cells were analyzed by flow cytometry and the Beckman-Coulter's Expo32 Multicom software. The Ig isotypes were identified with a rapid test paper (Roche, Mannheim, Germany), according to the method provided by the manufacturer. The competition assay showed that MABs 10F5

and 8C10 identified different epitopes. The L929/TLT-2 cells (1×10^6) were incubated with 10F5 and 8C10 (0 µg/10 µL, 0.5 µg/10 µL, 1 µg/10 µL, 2 µg/10 µL) for 45 min at 4°C. After being washed with PBS, the cells were incubated with 1 µg biotinylated identical MABs, followed by streptavidin-PE for 30 min for flow cytometry to ascertain the saturating amounts of each MAB.

Flow cytometric analysis

For flow cytometry, the cells mentioned above (1×10^6 /mL) were incubated with the MABs 10F5 and 8C10, respectively, for 30 min at 4°C and washed. For indirect staining, PE-labeled goat anti-mouse antibody as secondary antibody was incubated for another 30 min at 4°C and washed. The results were analyzed by flow cytometry facility and Beckman-Coulter's Expo 32 multicom software.

Dot-blot and Western blot analysis

To further analyze the binding ability of the antibodies to TLT-2 protein, the transfectants were lysed by RIPA Lysis Buffer (Cell Signaling Technology, Boston, MA) in an ice incubator for 2 h. Next, dot-blot and Western blotting assay were used to analyze the binding ability of the two MABs to recombinant TLT-2. Briefly, drops of the lysates were spotted onto circled dot (5 µm in diameter) on nitrocellulose membranes (Bio-Rad, Hercules, CA). After 30 min of drying at room temperature (RT), the membrane was blocked for 2 h at RT with 5% non-fat dry milk in PBS (pH 7.2) on a rocking platform. The membrane was then washed three times with PBS containing 0.1% Tween-20 (PBST), and incubated with PBS, 5 µg/mL 8C10, or 10F5 or mouse IgG1 for 2 h at RT. After being washed, membrane was sealed in 5% milk containing 1:2000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and incubated at RT for 1 h with agitation. The membrane was subjected to enhanced chemiluminescence kit (ECL, Roche) and then exposed to film.

For Western blotting, 10 µg of lysates was mixed with loading buffer and boiled at 95–100°C for 5 min followed by separating on 10% SDS-polyacrylamide gels under reducing conditions. Gel-loaded proteins were transferred onto a nitrocellulose membrane, which was treated as described above for dot-blot. Western blot analysis was performed to analyze the binding capacity of 10F5 and 8C10 MABs to TLT-2 protein on L929/TLT-2. L929/mock cells were set as negative control.

FIG. 4. Immunofluorescence analysis. The results showed that antibody 10F5 and 8C10 could identify the L929/TLT-2 gene transfection cell surface expression of TLT-2 molecules, but could not identify L929/mock gene transfected cells.

FIG. 5. Mutual competition assay of the MAbs. (A.1) After L929/TLT-2 cells were incubated with unlabeled 10F5 (0 µg/10 µL, 0.5 µg/10 µL, 1.0 µg/10 µL, 2.5 µg/10 µL), respectively, at 4°C for 45 min, cells were stained with the biotinylated identical MAb at 1 µg/test followed by staining of streptavidin-PE (grey histograms). (A.2) L929/TLT-2 transfectants were stained with saturating amounts (2.5 µg) of 10F5 MAb at 4°C for 30 min; then biotinylated 8C10 (0 µg/10 µL, 0.5 µg/10 µL, 1.0 µg/10 µL, 2.5 µg/10 µL) was added followed by supplement of streptavidin-PE (grey histograms). (B.1) L929/TLT-2 cells were incubated with unlabeled 8C10 (0 µg/10 µL, 0.5 µg/10 µL, 1.0 µg/10 µL, 2.5 µg/10 µL), respectively, at 4°C for 45 min; then cells were stained with the biotinylated identical MAb at 1 µg/test followed by staining of streptavidin-PE (grey histograms). (B.2) L929/TLT-2 transfectants were stained with saturating amounts (2.5 µg) of 8C10 MAb at 4°C for 30 min, then biotinylated 10F5 (0 µg/10 µL, 0.5 µg/10 µL, 1.0 µg/10 µL, 2.5 µg/10 µL) was added followed by supplement of streptavidin-PE (grey histograms). The fluorescence intensity of the stained cells was analyzed by flow cytometry. At the same time, L929/TLT-2 cells also stained with biotinylated mouse IgG1 followed by streptavidin-PE as negative control (open histogram). The figure is representative of eight independent experiments.

Immunofluorescence analysis

For immunofluorescence analysis, the cells mentioned above (1×10^6 /mL) were incubated with the MAbs 10F5 and 8C10, respectively, for 30 min at 4°C and washed. For indirect staining, PE-labeled goat anti-mouse antibody as secondary antibody was incubated for another 30 min at 4°C and washed. The results were analyzed by fluorescence microscope.

Analysis of T cell proliferation

Seeded peripheral blood mononuclear cells (PBMCs) were plated into 96-well plates pre-coated with LPS (100 ng/mL), anti-CD3 MAb (0.5 µg/mL) + IgG (1 µg, 2.5 µg, 5 µg, 10 µg/well), and anti-CD3 MAb (0.5 µg/mL) + 8C10 (1 µg, 2.5 µg, 5 µg, 10 µg/well) and cultured at 37°C, 5% CO₂. After 3 days in culture, the reagent of cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was added to the culture system and incubated for 6 h. The absorbance of every well was measured at 450 nm by a microplate reader (Bio-Rad) using enzyme-linked immunosorbent to assay the concentration of IL2, IL10, and INF-γ in culture supernatant. The procedure was performed as described previously.⁽²⁶⁾ Student's *t* test was applied for statistical analysis among the groups.

Results

Establishment of two novel anti-human TLT-2 MAbs

As shown in Figure 1, the L929/TLT-2 transfectants expressed on their membrane high amounts of target molecules in comparison with L929/mock cells. To generate MAbs specific for human TLT-2, the splenocytes of mice immunized by L929/TLT-2 cells were fused with myeloma SP2/0 cells. After repeated screening and cloning, two MAbs, named 10F5 and 8C10, were obtained. Figure 2 shows that 10F5 and 8C10 could recognize transfectant cells L929/TLT-2, but not L929/mock, L929/CD28, and L929/B7-H3 cells, indicating that 10F5 and 8C10 are specific for TLT-2. The isotype of 10F5 and 8C10 were IgG1 (κ).

For further analysis, we conducted Western blotting and dot-blot to confirm the specificity of the two MAbs and explore whether structural folding is indispensable for their recognition of TLT-2. As shown in Figure 3, only 10F5 MAbs could recognize the denatured TLT-2 protein, and both MAbs were proven in dot-blot to specifically bind to natural TLT-2 protein as compared with PBS and mouse IgG1 staining (negative control). The MAbs were also identified by immunofluorescence. The results indicated that both 10F5 and 8C10 can identify the L929/TLT-2 gene transfection cell surface expression of TLT-2 molecules, but cannot identify L929/mock gene transfected cells (Fig. 4). Thus, the two novel MAbs against human TLT-2 reported in this work provide additional reagents for the functional characterization of TLT-2.

MAbs 10F5 and 8C10 recognized different epitope

Next, competition assay was performed to further determine whether the two antibodies recognize the same, overlapping, or distinct epitopes of human TLT-2 antigen. At first the L929/TLT-2 cells (1×10^6) were incubated with 10F5 (0 µg/10 µL, 0.5 µg/10 µL, 1 µg/10 µL, 2.5 µg/10 µL) for 45 min at 4°C. After being washed with PBS, the cells were followed by staining with the detecting amounts (1 µg/test) of

biotin-conjugated identical antibody to ascertain the saturating concentration at which all binding sites on L929/TLT-2 were occupied by this MAb. As shown in Figure 5A, graded amounts of unlabeled 10F5 blocked subsequent binding of biotinylated MAb to L929/TLT-2 cells in a dose-dependent way and 2 μ g was sufficient for antibody to saturate their

interacting sites in TLT-2, respectively. Secondly, competition assay between 10F5 and 8C10 was performed using the same protocol. The results showed that 8C10 could not be blocked in the 10F5 saturated L929/TLT-2 cells (Fig. 5B). Taken together, these data indicated that these two MAbs (10F5 and 8C10) seemed to recognize different epitopes.

FIG. 6. Expression of TLT-2 antigen on different cell lines and immune cells was determined by FCM with MAbs 10F5 and 8C10. Different human cell lines were stained with MAbs (10F5, 8C10; grey histograms) or negative control mouse IgG (open histograms) at 4°C for 30 min, followed by goat anti-mouse IgG-phycoerythrin. (A) TLT-2 expression on tumor cell lines (Raji, Daudi, THP-1, Jurkat, M431, M231, Hct, SW480, and HeLa). (B) TLT-2 expression on immune cells (CD3⁺T, B, and monocytes). The figure is a representative of two experiments.

FIG. 7. Mixed lymphocyte proliferation upon engagement of TLT-2 with two MABs. Only MAB 8C10 inhibited T cell proliferation and inhibited the function of T cell in a dose-dependent manner. PBMC cells were seeded to the 96-well plates pre-coated with anti-CD3 MAB (0.5 mg/mL) and LPS (100 ng/mL). Meanwhile, different concentrations (1, 2.5, 5, or 10 mg/mL) of 8C10 MAB, 10F5, or mIgG1 isotype control were added to the culture systems for 72 h. T cell proliferation was determined by the CCK-8 reagent according to the protocol. The results are shown as the mean SD of triplicate wells (* $p < 0.05$ compared to the control groups). (A) T cell proliferation; (B–D) enzyme-linked immunosorbent to assay the concentration of IL2, IL10, and INF- γ in culture supernatant.

Expression of TLT-2 on different cell lines and immune cells

The expression of TLT-2 was examined on different human tumor cell lines using flow cytometry (Fig. 6). Results showed that TLT-2 could be highly expressed on tumor cell lines of Raji, Daudi, THP-1, monocytes, B cells, and activated T cells; weakly expressed on M431, M231, and T cells; but not expressed on Jurkat, Hct, SW480, Hela; also the two MABs we generated could be used in FCM analysis. With the expression of TLT-2 in these tumor cell lines detected by these new MABs 8C10 and 10F5, it may be assumed these two MABs may have great value for further study of the tumor stem cell.

8C10 inhibits T cell proliferation

To assess whether the blocking MABs 8C10 exhibited any functional effect on T cells, an *in vitro* direct proliferative assay was performed in the presence of MABs 10F5 or 8C10. Compared with the control groups, the addition of MABs 8C10 inhibited T cell proliferation and inhibited the function of T

cell in a dose-dependent manner that could be measured by CCK-8 and ELISA within 72 h of incubation (Fig. 7). Therefore, MABs 8C10 is a functional antibody that can inhibit TLT-2 positive signals on T cells.

Discussion

The fluctuant balance between co-stimulatory and co-inhibitory signals that a T cell receives participates in the initiation, effectivity, and termination of an immune response.^(27,28) Excessive activation and immune reaction of T cells may result in autoimmune diseases and host immune injury. TLT-2 was elucidated widely and distinctly expressed on many tissues and tumor cell lines. The significance of TLT-2 expression on tumor cells and the underlying mechanisms need further investigation. Therefore, the specific MABs against TLT-2 molecule, valuable tools for modulation of TLT-2 positive signal pathway, not only provides a new perspective for a thorough understanding of the delicate regulation of the immune system, but also plays a potential role in clinical application.

Although several articles have reported on the generation of monoclonal antibodies against human TLT-2, only two of these were found to reveal agonistic or antagonistic functions.⁽²⁴⁾ Thus developing more efficient and specific monoclonal antibodies may be important for further disclosure of TLT-2. The choice of an appropriate immunization strategy and screening assay is the most important part of hybridoma production. In order to avoid an immune response against heterogeneous antigen in this study, mouse original fibrous cell line L929 was selected to carry the target antigen. Human TLT-2 antigen presented by the L929 cells can produce a more specific immune response and decrease the probability of non-specific MAb generation. All the hybridomas were screened by flow cytometry, whose constraints on screening of assays are reliable, fast, and simple. According to these considerations, we successfully obtained two desired hybridomas secreting mouse anti-human TLT-2 MAbs by one fusion operation.

After purification, the two novel mouse MAbs against human TLT-2 were generated, identified, and subjected to strict verification by immunofluorescence dot-blot and Western blot analysis. Both 10F5 and 8C10 specifically bound to natural TLT-2 protein in dot-blot and immunofluorescence while in conditioned Western blotting the denatured TLT-2 protein could be immunoblotted with 10F5. This indicated that 10F5 could bind to a linear epitope. Competition assay showed that the two MAbs recognize different epitopes of TLT-2.

Additional experimental evidence showed that TLT-2 could be highly expressed on tumor cell lines of Raji, Daudi, THP-1, monocytes, B cells, and activated T cells; weakly expressed on M431, M231, and T cells; and not expressed on Jurkat, Hct, SW480, and Hela. Moreover, the blocking MAbs 8C10 exhibited functional effect on T cells. Engagement of human TLT-2 by the 8C10 MAbs could inhibit T cell proliferation, suggesting that 8C10 may be useful for blocking the TLT-2 positive signal.

With their high specificity and few side effects, monoclonal antibodies have recently been unanimously accepted as ideal medicaments in targeting therapy in some refractory diseases.⁽²⁹⁾ With the expression of TLT-2 on the cells above, detected by the new MAbs 10F5 and 8C10, these two MAbs may have great value for the further study of diseases. Therefore, 10F5 and 8C10 have a potential value in further studying tumor escape, autoimmune diseases, and infectious diseases.

In conclusion, we have prepared two specific mouse MAbs against human TLT-2. They recognize different epitopes. One of the MAbs acts as an adjuvant to inhibit proliferation on purified human T cells. This makes 8C10 a valuable tool for further exploration of TLT-2 function. Furthermore, another anti-TLT-2 MAb will be generated, so a sandwich ELISA technology may be developed based on these MAbs to analyze TLT-2 in view of its important regulatory functions.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (grant no. 30901313).

Author Disclosure Statement

The authors have no financial conflict of interest to disclose. The generated antibodies were distributed solely to non-profit research organizations for research purposes only.

References

1. Natarajan K, Dimasi N, Wang J, Mariuzza RA, and Marquies DH: Structure and function of natural killer cell receptors: multiple molecular solutions to self, nonself discrimination. *Annu Rev Immunol* 2002;20:853–885.
2. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, and Aderem A: The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *PNAS* 2000;97:13766–13771.
3. Navarro F, Llano M, Bellon T, Colonna M, Geraghty DE, and López-Botet M: The TLT2(LIR1) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cells. *Eur J Immunol* 1999; 29:277–283.
4. Rajagopalan S, Winter CC, Wagtmann N, and Long EO: The Ig-related killer cell inhibitory receptor binds zinc and requires zinc for recognition of HLA-C on target cells. *J Immunol* 1995;55:4143–4146.
5. Medzhitov R: Toll-like receptor and innate immunity. *Nat Rev Immunol* 2001;1:135–145.
6. Akira S, Takeda K, and Kaisho T: Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Rev Immunol*, 2001;2:675–680.
7. Wilson M J, Torkar M, Haude A, Milne S, Jones T, Sheer D, Beck S, and Trowsdale J: Plasticity in the organization and sequences of human KIR/ILT gene families. *PNAS* 2000;97: 4778–4793.
8. Torkar M, Haude A, Milne S, Beck S, Trowsdale J, and Wilson MJ: Arrangement of the ILT gene cluster: a common null allele of the ILT6 gene results from a 6.7-kbp deletion. *Eur J Immunol* 2000;30:3655–3662.
9. Martin AM, Freitas EM, Witt CS, and Christiansen FT: The genomic organization and evolution of the natural killer immunoglobulin-like receptor (KIR) gene cluster. *Immunogenetics* 2000;51:268–280.
10. Bahram S, Bresnahan M, Geraghty DE, and Spies T: A second lineage of mammalian major histocompatibility complex class I genes. *PNAS* 1994;91:6259–6263.
11. Radosavljevic M, Cuillerier B, Wilson MJ, Clément O, Wicker S, Gilfillan S, Beck S, Trowsdale J, and Bahram S: A cluster of ten novel MHC class I related genes on human chromosome 6q24.2–q25.3. *Genomics* 2002;79:114–123.
12. Lanier LL, and Bakker AB: The ITAM-bearing transmembrane adaptor DAP-12 in lymphoid and myeloid cell function. *J Immunol* 2000;12:611–614.
13. Allcock RJ, Barrow AD, Forbes S, Beck S, and Trowsdale J: The human TREM gene cluster at 6p21.1 encodes both activating and inhibitory single IgV domain receptors and includes NKp44. *Eur J Immunol* 2003;33:567–577.
14. Bouchon A, Dietrich J, and Colonna M: Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol* 2000;10:4991–4995.
15. King RG, Herrin BR, and Justement LB: Trem-like transcript 2 is expressed on cells of the myeloid/granuloid and B lymphoid lineage and is up-regulated in response to inflammation. *J Immunol* 2006;176:6012–6021.
16. Halpert MM, Thomas KA, King RG, and Justement LB: TLT2 potentiates neutrophil antibacterial activity and chemotaxis in response to G protein-coupled receptor-mediated signaling. *J Immunol* 2011;87:2346–2355.
17. Kobori H, Hashiguchi M, Piao J, Kato M, Ritprajak P, and Azuma M: Enhancement of effector CD8+ T-cell function by

- tumour-associated B7-H3 and modulation of its counter-receptor triggering receptor expressed on myeloid cell-like transcript 2 at tumour sites. *J Immunol* 2010;130:363–373.
18. Leitner J, Klauser C, Pickl WF, Stockl J, Majdic O, Bardet A, Kreil DP, Dong C, Yamazaki T, Zlabinger G, Pfistershammer K, and Steinberger P: B7-H3 is a potent inhibitor of human T-cell activation: no evidence for B7-H3 and TREM2 interaction. *Eur J Immunol* 2009;39:1754–1764.
 19. Tran CN, Thacker SG, Louie DM, Oliver J, White PT, Endres JL, Urquhart AG, Chung KC, and Fox DA: Interactions of T cells with fibroblast-like synoviocytes: role of the B7 family costimulatory ligand B7-H3. *J Immunol* 2008;180:2989–2998.
 20. Zhang G, Hou J, Shi J, Yu GB, and Zhang X: Soluble CD276(B7-H3) is released from monocytes, dendritic cells and activated T cell and is detectable in normal human serum. *Immunology* 2008;123:538–546.
 21. Sun J, Chen LJ, Zhang GB, Jiang JT, Zhu M, Tan Y, Wang HT, Lu BF, and Zhang XG: Clinical significance and regulation of the costimulatory molecule B7-H3 in human colorectal carcinoma. *Cancer Immunol Immunother* 2010;59:1163–1171.
 22. Zhang G, Wang J, Kelly J, Gu G, Hou J, Zhou Y, Redmond HP, Wang JH, and Zhang X: B7-H3 augments the inflammatory response and is associated with human sepsis. *J Immunol* 2010;185:3677–3684.
 23. Masaaki A, Hashiguchi M, Hiroko H, Kamimura Y, Kozono H, and Azuma M: Triggering receptor expressed on myeloid cell-like transcript (TLT-2) is a counter-receptor for B7-H3 and enhances T cell responses. *PNAS*. 2008;30:10495–10500.
 24. Hashiguchi M, Inamochi Y, Nagai S, Ostuki N, Piao J, Kobori H, Kanno Y, Kojima H, Kobata T, and Azuma M: Human B7-H3 binds to triggering receptor expressed on myeloid cells-like transcript 2 (TLT-2) and enhances T cell responses. *Open J Immunol* 2012;2:9–16.
 25. Qiu YH, Sun ZW, Shi Q, Su CH, Chen YJ, Shi YJ, Tao R, Ge Y, and Zhang XG: Apoptosis of multiple myeloma cells induced by agonist monoclonal antibody against human CD28. *Cell Immunol* 2005;236:154–160.
 26. Chen YJ, Hu ZH, Wang Q, Ge Y, Bai LX, Wang XF, and Zhang X: Generation and characterization of four novel monoclonal antibodies against human programmed death-1 molecule. *Hybridoma* 2010;29:153–160.
 27. Lenschow DJ, Walunas TL, and Bluestone JA: CD28/B7 system of T cell costimulation. *Annu Rev Immunol* 1996;14:233–258.
 28. Greenwald RJ, Freeman GJ, and Sharpe AH: The B7 family revisited. *Annu Rev Immunol* 2005;23:515–548.
 29. Dotti G: Blocking PD-1 in cancer immunotherapy. *Blood* 2009;114:1457–1458.

Address correspondence to:

Dr. Xueguang Zhang
 Institute of Medical Biotechnology
 Soochow University
 708, Renmin Road
 Suzhou, Jiangsu, 215007
 P.R. China

E-mail: xueguangzh@yahoo.com.cn

Received: September 24, 2012

Accepted: March 4, 2013