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CUTTING EDGE

Cutting Edge: The Mouse NK Cell-Associated Antigen Recognized by DX5 Moncoclonal Antibody is CD49b (α_2 Integrin, Very Late Antigen-2)[1]

Hisashi Arase,* Takashi Saito,† Joseph H. Phillips,‡ and Lewis L. Lanier[2]*

DX5 mAb is a useful reagent because it stains NK cells from all mouse strains examined. We have identified the molecule recognized by DX5 mAb by using a retrovirus-mediated expression cloning system. A 5-kb cDNA encoding a protein that is reactive with the DX5 mAb was isolated from a NK cell cDNA library, and this molecule was identical with CD49b (very late Ag-2, α_2 integrin). The DX5 mAb reacted with transfectants expressing CD49b, and binding of DX5 to the NK cells and CD49b transfectants was blocked in the presence of other anti-CD49b mAbs. When NK1.1$^+$ NK cells were cultured with IL-2, they progressively lost reactivity with DX5 mAb as a consequence of cellular proliferation. Cytotoxicity mediated by the DX5$^+$ NK cells was dramatically higher as compared with DX5$^-$ NK cells. Therefore, DX5 mAb recognizes CD49b and can be used to define functionally distinct subsets of NK cells. ***The Journal of Immunology*****, 2001, 167: 1141–1144.**

Natural killer cells play an important role in innate immunity by killing tumors and virus-infected cells (1–3). The identification of NK cells and the study of their function has been made possible by the use of mAbs that react with Ags preferentially expressed on their cell surface. The prototypic NK cell Ag is NK1.1 (NKR-P1C), a molecule expressed on all NK cells but rarely found on T cells, except for NKT cells (4, 5). However, NK1.1 is an alloantigen whose expression is limited to NK cells in a only a few inbred mouse strains (e.g., C57BL/6 or C57BL/10); most other inbred mouse strains do not express NK1.1 (6). Previously, we generated a mAb, designated DX5, that brightly stains NK cells and a small subset of T cells (partially, but not exclusively, overlapping with NKT cells) in all strains of mice that have been analyzed (S. Lazetic and J. H. Phillips, unpublished observations). Therefore, DX5 mAb has been a useful reagent to identify and isolate NK cells from mice lacking expression of the NK1.1 Ag (for example, in gene-deficient mice generated on the 129 background) (7, 8). Although the DX5 mAb is widely used as a pan-NK cell marker by many laboratories, the molecular nature of the Ag has not previously been known. In this study, we define the specificity of the Ag recognized by the DX5 mAb and show that DX5 can be used to define function subsets of NK cells in vitro.

Materials and Methods

NK cell preparation

NK cells were purified from C57BL/6 or BALB/c mice as previously described (9). Briefly, splenocytes were depleted of CD4-, CD8-, and surface Ig-positive cells by magnetic cell sorting and were then stained with PE-DX5 mAb (BD PharMingen, San Diego, CA), followed by incubation with magnetic microbeads coated with anti-PE-Ab (Miltenyi Biotec, Bergisch Gladbach, Germany). Thereafter, DX5$^+$ cells were isolated by magnetic cell sorting using a MACS (Miltenyi Biotec). The purified NK cells were used for construction of a cDNA library or were cultured in RPMI 1640 supplemented with 10% FCS and 5×10^{-5} M 2-ME in the presence of 4000 U/ml human rIL-2 (generously provided by the National Cancer Institute (Preclinical Repository, Biological Resources Branch, Frederick, MD) for the indicated period.

Construction of the cDNA library

Poly(A)$^+$ RNA was isolated from freshly purified DX5$^+$ BALB/c NK cells using a mRNA purification kit (Amersham Pharmacia Biotech, Piscataway, NJ). The cDNAs were made from the purified mRNA using the SuperScript plasmid system (Life Technologies, Rockville, MD). The cDNAs were ligated into the pMxs retrovirus vector, a variant of the pMX retroviral vector (10) in which the multicloning site was modified to permit ligation of the cDNA inserts. Thereafter, ElectroMAX DH5α competent cells (Life Technologies) were transformed with the cDNA library. The complexity of the cDNA library was $\sim 1 \times 10^6$.

Library screening

The cDNA library was transfected into Plat-E packaging cells (11) using LipofectAMINE PLUS (Life Technologies). Two days later, supernatant containing viruses was collected and used to infect 1×10^7 mouse T cell hybridoma cells (I-E^k-restricted T cell hybridoma recognizing pigeon cytochrome *c*) in the presence of *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate DOTAP (10 μg/ml; Roche Diagonostic Systems, Somerville, NJ). Two days later, DX5$^+$ cells were purified by magnetic sorting using PE-DX5 mAb and magnetic microbeads conjugated with anti-PE Ab, using a procedure similar to the method used to purify NK cells. Purification of DX5$^+$ cells was repeated six times within the interval of 2 or 3 days. Thereafter, T cell hybridomas reactive with the DX5 mAb were cloned by limiting dilution. The cDNAs derived from the retroviral library were amplified from the genomic DNA of DX5$^+$ T hybridoma clones by

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PCR using oligonucleotide primers designed for the retroviral vector sequence (5′-GGTGGACCATCCTCTAGACT and 3′-CCCTTTTT CTGGAGACTAAAT).

Monoclonal Abs and flow cytometry

Lewis rats were immunized with NK cells isolated from C57BL/6 mice and were fused with the mouse SP2/0 myeloma cell line by using conventional techniques. The DX5 mAb is a rat IgM Ab selected for reactivity with mouse NK cells. The mAbs used for flow cytometry were: PE-anti-NK1.1, PE-DX5, PE-anti-CD49b (clone HMα2), FITC-anti-CD29, CyChrome-anti-CD3, and FITC-anti-heat stable Ag (HSA)[3] (generously provided by Dr. A. Stall, BD PharMingen). To analyze cell proliferation, freshly isolated NK cells suspended in PBS were labeled with CFSE (5 μM; Molecular Probes, Eugene, OR) for 8 min at room temperature, followed by extensive washing. Stained cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA).

Cytotoxicity assay

NK cell-mediated cytotoxicity was analyzed by using a standard ^{51}Cr-release assay, as previously described (12).

Results and Discussion

Expression cloning of the Ag recognized by DX5 mAb

A cDNA library in a retroviral vector was generated from DX5$^+$ NK cells isolated from BALB/c mice. A mouse T cell hybridoma was transduced with the retroviral library, and DX5$^+$ cells were purified by magnetic cell sorting. Most of the cells reacted with the DX5 mAb after six rounds of enrichment. Single-cell clones were obtained by limiting dilution, and cDNAs derived from the retroviral library were amplified by PCR using vector sequence primers. A 4.6-kb cDNA was amplified from one DX5$^+$ T hybridoma clone, and sequencing of this gene revealed that it is identical with mouse CD49b. Because CD49b is known to form a heterodimer with CD29 (β_1 integrin) (13), we stained the DX5$^+$ T cell hybridoma clones with the anti-CD49b, HMα2 mAb (14), and an anti-CD29 mAb. As shown in Fig. 1*A*, the DX5$^+$ T hybridoma clone, but not the parental T cell hybridoma, reacted with anti-CD49b mAb. Furthermore, CD29 expression was up-regulated on the DX5$^+$ T hybridoma clone compared with the parental T hybridoma. Similarly, freshly isolated NK cells from C57BL/6 mice were stained with both DX5 and HMα2 mAbs (Fig. 1*B*). Staining of NK cells by DX5 mAb was completely blocked by preincubation of NK cells with anti-CD49b HMα2 mAb. These observations confirm that DX5 mAb recognizes CD49b and that the epitope recognized by DX5 is close to that bound by HMα2 mAb.

When CD49b cDNA was transduced by retroviral infection into various tumor cells (e.g., mouse Ba/F3 pro-B cells, mouse B16 melanoma cells, human 293T cells, and human Jurkat T cells), transduced cells expressing very high levels of mouse CD49b all stained with both the DX5 and HMα2 mAbs. However, we observed that transduced cells expressing lower amounts of CD49b, as detected by the HMα2 mAb, were often either dimly stained or not reactive with the DX5 mAb. This is illustrated by the series of Jurkat cell clones shown in Fig. 1*C*. These results indicate that DX5 mAb can recognize CD49b only when it is present in substantial amounts on the cell surface. HMα2 is a hamster IgG mAb, whereas DX5 is a rat IgM. In general, IgGs have a higher affinity for Ag than IgMs. Therefore, this difference in the isotype between these mAbs may be responsible for the difference in their ability to stain the cells expressing low levels of CD49b. However, we cannot formally exclude that these differences might be due to post-translational modification of the Ag on a fraction of CD49b that prevents efficient binding of the DX5 mAb. Treatment with neuraminidase to remove terminal sialic acid did not affect staining with the DX5 mAb (H. Arase, unpublished observation). CD49b is not expressed on most of splenic T cells in vivo, including CD44$^+$ memory T cells, and is present on only a subset of NKT cells (Ref. 15 and our unpublished observation). In addition, the expression level of CD49b on NKT cells is lower than on NK cells (data not shown). The ability of DX5 mAb to detect cells expressing relatively high levels of CD49b might be advantageous in using this reagent to discriminate NK cells from other CD49b-bearing cells.

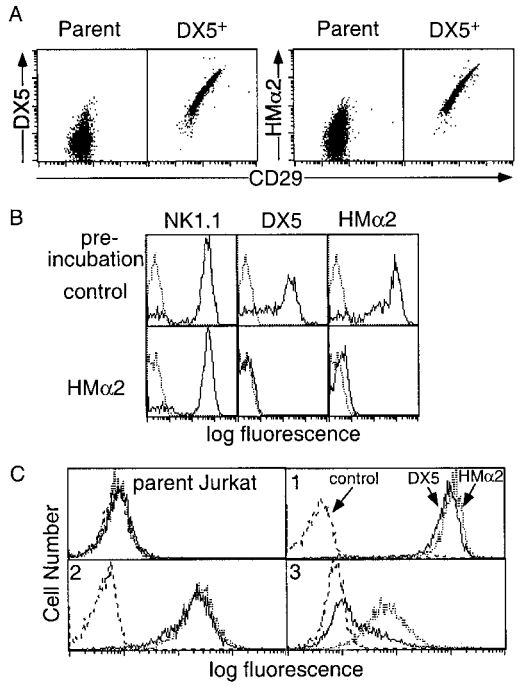

FIGURE 1. Recognition of CD49b by DX5 mAb. *A*, The parental mouse T cell hybridoma and a DX5$^+$ T hybridoma clone obtained from cells transduced with the mouse NK cell retroviral library were stained with anti-CD29 mAb in combination with the DX5 or HMα2 mAbs. *B*, Splenocytes, depleted of B cells, were stained with anti-HSA and anti-CD3 mAbs in combination with anti-NK1.1, DX5, and HMα2 mAbs (solid line) or control (dotted line) as indicated. Histograms of cells, gated to exclude CD3$^+$ and HSA$^+$ cells (Ags that are not present on NK cells), are shown. *C*, Parental human Jurkat T cells and Jurkat clones transduced with mouse CD49b were stained with DX5 mAb (solid line), HMα2 mAbs (dotted line), or control (long dotted line). Representative clones with different densities of mouse CD49b Ag are shown.

DX5 mAb distinguishes two populations of IL-2-cultured NK cells

NK cells constitutively express IL-2R β- and γ-chains and can be expanded by using a high concentration of IL-2. However, after expansion in IL-2, ~20–30% of the NK cells, identified as CD3$^-$NK1.1$^+$ lymphocytes, were not stained by the DX5 mAb (Fig. 2*A*) but were recognized by the anti-CD49b HMα2 mAb. Because DX5 apparently does not recognize cells expressing low levels of CD49b, this difference in the staining pattern between DX5 and HMα2 mAbs may be due to the difference in the amount of CD49b expression. To determine the origin of the DX5-negative/dim (DX5$^-$) population that appears after culture with IL-2, freshly isolated NK cells were labeled with CFSE and the number of cell divisions was compared between DX5$^+$ and DX5$^-$ NK cells after 5 days of culture with IL-2. As shown in Fig. 2*B*, there was a progressive loss of DX5 Ag that directly correlated with cell division. This suggested that the DX5$^-$ cells expanded four to

[3] Abbreviations used in this paper: HSA, heat-stable Ag; DX5$^-$, DX5-negative/dim; YAC, yeast artificial chromosome.

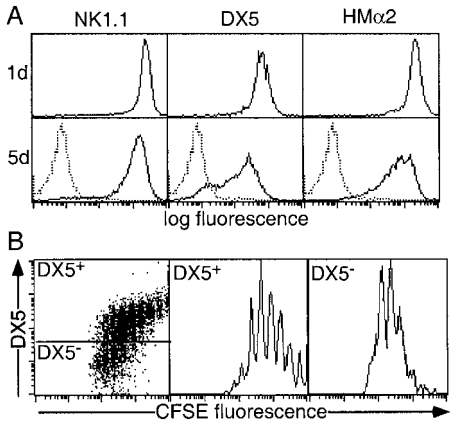

FIGURE 2. Appearance of DX5$^-$ cells in IL-2-expanded mouse NK cells. *A*, NK cells were cultured in the presence of IL-2 for the indicated periods. Thereafter, cells were stained with anti-NK1.1, DX5, and HMα2 mAbs (solid lines) or control (dotted lines). *B*, NK cells were labeled with CFSE and cultured in the presence of IL-2 for 5 days. Thereafter, cells were stained with PE-DX5 mAb, and the amount of CFSE remaining in the cells is shown for the DX5$^+$ (*center*) and DX5$^-$ (*right*) NK cells. A bivariate plot of DX5 mAb staining vs CFSE demonstrates progressive loss of CD49b Ag expression during cellular proliferation (*left*).

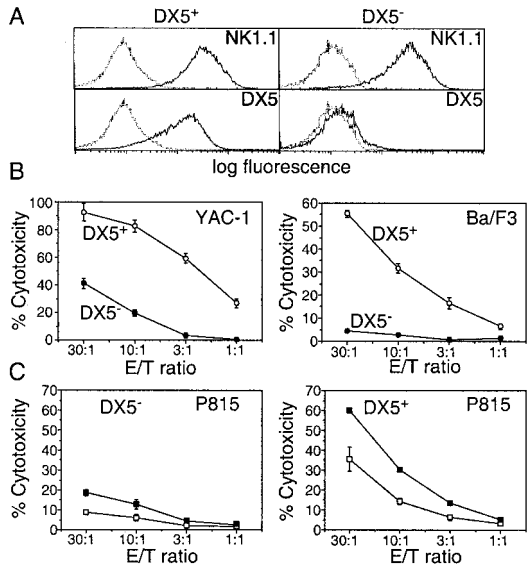

FIGURE 3. Purification and functional analysis of DX5$^+$ and DX5$^-$ NK cells. *A*, NK cells were cultured in the presence of IL-2 for 5 days, and DX5$^+$ and DX5$^-$ cells were separated by MACS. Three days later, these cells were stained with anti-NK1.1 and DX5 mAbs (solid lines) or control (dotted lines). *B*, Purified DX5$^+$ (○) and DX5$^-$ (●) NK cells were cultured for 2 days and were then used for cytotoxicity assays. Cytotoxicity against YAC-1 and Ba/F3 cells is shown. *C*, Redirected cytotoxicity assays using DX5$^+$ and DX5$^-$ NK cells as effectors against P815 targets were performed in the presence (■) or absence (□) of anti-NK1.1 mAb (final concentration, 10 μg/ml).

eight times more than the DX5$^+$ cells. Because contamination of the DX5$^+$ NK cell subset with DX5$^-$ NK cells was <1% before culture, these data indicated that most of the DX5$^-$ cells were derived form DX5$^+$ cells. Indeed, when freshly isolated splenocytes were stained with anti-CD3, anti-NK1.1, and DX5 mAbs, we could detect a small population that expressed NK1.1, but not DX5 and CD3 (data not shown). Because of the low frequency of these cells in the spleen, we were unable to isolate enough of these CD3$^-$NK1.1$^+$DX5$^-$ cells to study their function.

The cytotoxicity of in vitro-derived DX5$^+$ and DX5$^-$ NK cells was analyzed against several tumor cell lines. Purified NK cells were cultured for 5 days and were further purified into DX5$^+$ and DX5$^-$ cells by MACS. The purified cells were cultured for 2 days and used for cytotoxicity assays. Staining by DX5 mAb remained the same after 3 days of culture, and there was no significant difference in NK1.1 expression between the DX5$^-$ and DX5$^+$ cells (Fig. 3*A*). Surprisingly, DX5$^-$ cells showed only very weak cytotoxicity compared with DX5$^+$ cells against YAC-1 and Ba/F3 targets (Fig. 3*B*). Similar results were obtained when P388D1, RMA, and B16 cells were used as targets (data not shown). Abs against NK1.1 are able to trigger NK cell-mediated killing of FcR-bearing target cells such as P815 (12). In this assay, DX5$^-$ cells also showed weak anti-NK1.1-meditated re-directed cytotoxicity against P815 targets, although the expression level of NK1.1 was the same on the DX5$^+$ and DX5$^-$ NK cells. These data suggested that the DX5$^-$ NK cells may be deficient in their lytic function.

Function of CD49b on NK cells

CD49b (α_2 integrin) is known to bind to collagen or laminin (16). Moreover, CD49b is expressed on many tissues, including platelets, where it plays an important role in their activation by damaged tissue (17). Indeed, platelets can be well stained with DX5 mAb (our unpublished observation). Although CD49b has been described on in vitro-cultured human peripheral blood NK cells, expression of CD49b on murine NK cells has not been reported, and its function has not been examined. Because the anti-CD49b HMα2 mAb has been reported to block the binding of CD49b to collagen (14), we examined the effect of this mAb as well as DX5 on NK cell adhesion. However, binding of NK cells to collagen-coated plates was not blocked by HMα2 or DX5 mAb (data not shown), suggesting that other collagen receptors may participate in the binding of NK cells to this extracellular matrix protein. In addition, NK cells did not induce redirected cytotoxicity using HMα2 (an IgG mAb) against FcR-positive P815 cells. No significant IFN-γ production was observed upon stimulation with immobilized anti-CD49b mAb, and there was no augmentation in cytokine production when NK1.1 and CD49b were simultaneously cross-linked (H. Arase, unpublished observations). Finally, cytotoxicity of NK cells against YAC-1 was not blocked by addition of the DX5 or HMα2 mAb. Therefore, the function of CD49b on NK cells remains unclear.

In contrast to mouse splenic NK cells, freshly isolated human peripheral blood NK cells do not express CD49b (18). Also unlike the situation with mouse NK cells, CD49b is apparently up-regulated, rather than down-regulated, when human NK cells are cultured in vitro with IL-2 (19, 20). Furthermore, human NK cells have been reported to be activated when cultured with immobilized anti-CD29 mAb (21). Whether these differences between mouse and human NK cells are species-specific or due to the reagents and assays used isn't clear. Further studies, ideally in mice with disrupted *CD49b* genes, are necessary to reveal the physiological role of this integrin in NK cell differentiation and effector function.

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