

THY-1⁺ AND THY-1⁻ NATURAL KILLER CELLS
Only Thy-1⁻ Natural Killer Cells Suppress Dendritic Cells

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Cells highly enriched for NK activity suppress the one-way mixed lymphocyte (ML) and the autologous mixed lymphocyte (AML) reactions by eliminating or suppressing dendritic cells (DC), which are required for stimulating lymphocyte proliferation (1). NK cells contain two phenotypically and functionally different populations; one population is Thy-1⁺ and the other is Thy-1⁻. We have used the FACS to obtain nearly homogeneous populations of Thy-1⁺ and Thy-1⁻ NK cells and we find that the two populations differ in their target specificity; only Thy-1⁻ NK cells suppress or eliminate DC and in this way regulate lymphocyte proliferation.

Materials and Methods

Preparations of Cells and Cultures. RPMI-1640 with 25 mM HEPES and L-glutamine supplemented with 2 mM L-glutamine, 5×10^{-5} M 2-ME, 10% FCS, 5,000 U of penicillin, 5 mg of streptomycin, and 1.5 mg of gentamycin per 100 ml of medium were used for all cell preparations. Cells were enriched for NK activity from C3H mice as previously described (1, 2). DC were prepared by incubating 10^6 C3H fresh whole spleen cells suspended in 10 ml of medium in 100-mm petri dishes for 2 h; nonadherent cells were removed by gentle pipetting and washing plates three times to remove the vast majority of lymphocytes; the adherent cells were incubated for an additional 22 h. Cells that detach during this incubation are predominantly DC and represent ~1–2% of all nucleated spleen cells (3) and for convenience are referred to as DC. Responder cells were whole spleen cells treated with carbonyl iron particles and then with anti-DC antibody (see below) and complement. DC (10^5 /well) and responder cells (10^6 /well) in 150 μ l medium were mixed with either 50 μ l medium or 50 μ l containing NK cells. Cultures were in 96-well plates and were immunized with 4×10^6 SRBC in a volume of 10 μ l. PFC were enumerated as previously described (2). The number of direct PFC per culture is the mean of counts on four aliquots of a pool prepared from three replicate cultures \pm SEM.

Chromium Release Assay. 1.25×10^5 YAC-1 or P815 target cells, instead of 5×10^3 target cells, were used to conserve effector cells that could be recovered only by cell sorting; otherwise assays were done as previously described (1).

Antibodies and Complement. Anti-Thy-1 (AT83A) and anti-DC antibody (33D1) (4) were kind gifts from Drs. F. W. Fitch and R. M. Steinman, respectively; anti-Ly-1.1, anti-Ly-2.1, and anti-I-E^k mAbs and low-tox-M rabbit complement (C') were purchased from Accurate Chemical and Scientific Corp., Westbury, NY. Anti-asialo GM1 antiserum (anti-AGM1) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. 10^7 cells/ml medium without FCS were treated with anti-Thy-1, final dilution (1:10); anti-Ly-

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1.1, final dilution (1:20); anti-Ly-2.1, final dilution (1:20); anti-I-E^k, final dilution (1:10); or anti-AGM1, final dilution (1:100) and C', final dilution (1:10) for 1 h at 37°C. Cells were washed twice after treatment.

Immunofluorescent Staining and FACS Analysis. Anti-AGM1 antiserum was purified over a CM Affi-Gel Blue column (Bio-Rad Laboratories, Richmond, CA). The IgG fraction was concentrated with polyethylene glycol (Cat. No. P-2263; Sigma Chemical Co., St. Louis, MO), dialyzed, and biotinylated (anti-AGM1-b). Purified anti-Thy-1 was prepared from ascites by (NH₄)₂SO₄ precipitation, followed by dialysis against 0.05 M borate, 0.04 M NaCl at pH 9.3. FITC was conjugated to anti-Thy-1 according to the procedure of Research Organics Inc., Cleveland, OH (Cat. No. 4027F) (5). Before staining, the antibodies were centrifuged for 5 min at 10,000 g; the anti-Thy-1-FITC was diluted 1:3,000 and the anti-AGM1-b 1:50 for staining. Avidin phycoerythrin (AvPe) (Becton Dickinson Immunocytometry Systems, Mountainview, CA) was used at a dilution of 1:20. The reagents were diluted in PBS containing 1.0% BSA and 0.1% azide. For analyses, 10⁵ cells were incubated for 15 min with anti-AGM1-b, washed three times, and resuspended in AvPe or a mixture of AvPe and anti-Thy-1-FITC. The samples were then incubated for 15 min in the dark and washed three times. For sorting, cells were stained with anti-Thy-1-FITC in PBS containing 10% FCS. 488 nm excitation was used for sorting (FACS IVB; Becton Dickinson Immunocytometry Systems). ~7 × 10⁶ cells were sorted per run and cell recovery was usually ~5 × 10⁵ Thy-1⁺ cells and 2 × 10⁶ Thy-1⁻ cells. Dead cells and debris were identified by propidium iodide uptake and were excluded from analysis. The 514 nm excitation was chosen during dual staining because the anti-AGM1-b/AvPe combination was too dull at 488 nm excitation. Propidium iodide emission was collected using a 620-nm-long pass filter. FITC and AvPe emissions were separated using a 560-nm dichroic mirror in combination with a 530/30-nm band pass filter for FITC, and a 575/25-nm band pass filter for AvPe. Lymphocytes were gated on forward light scatter; 20,000 viable cells per sample were collected for analysis. Cells were stained, sorted, and stored until used at 4°C.

Results

Enrichment for NK Activity. The progressive enrichment for cells cytotoxic for YAC-1 is shown in Fig. 1. Treatment with anti-Thy-1, anti-Ia, and C' causes additional enrichment of cells cytotoxic for YAC-1 but eliminates cytotoxicity for P815 cells (Fig. 1). In other experiments, the activity of NK cells for either YAC-1 or P815 was not affected by treatment with either anti-Ly-1.1, anti-Ly-2.1, or anti-L3T4 and C' (data not shown). If cells enriched for NK activity are treated with anti-AGM1 and C' rather than anti-Thy-1, anti-Ia, and C', then cytotoxicity for both YAC-1 and P815 cells is eliminated (Fig. 1). Thus, AGM1⁺ cells must include two populations: one that is Thy-1⁻ and cytotoxic for YAC-1 but not for P815, and another population that is Thy-1⁺ and cytotoxic for P815.

Isolation of Thy-1⁺ and Thy-1⁻ NK Cells. Cells from polyinosinic polycytidylic acid (poly I:C)-injected and x-irradiated mice were first treated with carbonyl iron particles and then were treated with anti-Ly-1.1, anti-Ly-2.1, anti-Ia antibodies, and C' to further reduce contaminating cells. The remaining cells were analyzed in two ways: (a) The cells were stained with biotinylated anti-AGM1 (AGM1-b) or simultaneously with anti-Thy-1-FITC and AGM1-b, and analyzed to determine the total percentage of AGM1⁺ cells and the percentage of AGM1⁺ and Thy-1⁺ cells; and (b) the cells were sorted into a strongly Thy-1⁺ population (the 14% of the total cells that stained most heavily) and into a population with fluorescence equivalent to background or autofluorescence (~35% of the total cells sorted). In the first kind of analysis, ~95% of the cells were AGM1⁺ of which ~33% were also Thy-1⁺ (data not shown). For the second type of analysis,

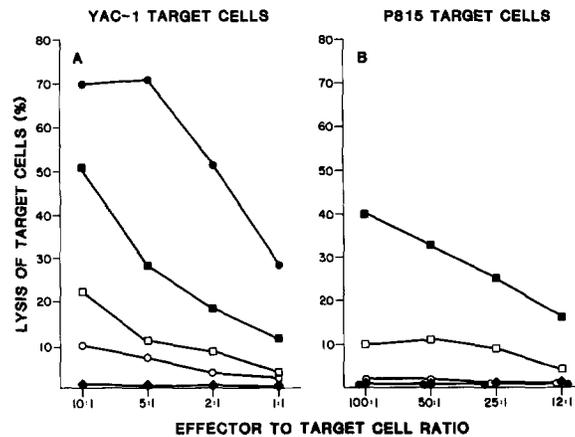


FIGURE 1. Progressive enrichment for NK activity. NK activity was assayed against YAC-1 or P815 target cells using spleen cells from: normal mice (○), mice injected with poly I:C -24 h (□), cells from mice injected with poly I:C -24 h and irradiated 12 h before killing; the spleen cells were treated with carbonyl iron particles to remove adherent cells (■); however, treatment of such cells with anti-Thy-1, anti-Ia, and C' causes additional enrichment for cells cytotoxic for YAC-1 but eliminates cytotoxicity for P815 cells (●). Treatment of cells with anti-AGM1 and C' eliminates all NK activity against either YAC-1 or P815 cells (◆). Note that the E/T ratios are different against YAC-1 and P815.

~93% of the cells sorted as strongly Thy-1⁺ were strongly Thy-1⁺ on rerun, and ~97% of the cells in the Thy-1⁻ sorted population on rerun had only background or autofluorescence. In addition, cells from both populations of sorted cells were prepared (Shandon Cytospin, Shandon Southern Instruments Inc., Sewickley, PA) and stained with Wright-Giemsa for light microscopy; >80% of the cells in both populations were lymphocyte-like cells with relatively few, ~5–20, large azurophilic granules. Thus, both the Thy-1⁺ and Thy-1⁻ cells had the morphology of cells frequently referred to as large granular lymphocytes.

The Cytotoxicity of Stained and Sorted NK Cells. The sorted Thy-1⁺ and Thy-1⁻ NK populations were assayed for cytotoxicity against YAC-1 and P815 target cells. Controls included NK cells that were unstained or stained with anti-Thy-1-FITC but not sorted. While both populations of NK cells (Thy-1⁺ and Thy-1⁻) were cytotoxic for YAC-1 target cells, only the Thy-1⁺ NK cells were cytotoxic for P815 cells (Fig. 2, A and C). Our previous studies have shown that NK cells lose cytotoxic activity within 24 h in culture (1). Fig. 2 (B and D) shows that both Thy-1⁺ and Thy-1⁻ NK cells lose lytic capacity during 24 h in culture.

NK Cells Suppress the B Lymphocyte Response by Suppressing or Eliminating DC. To show a critical role for DC in the PFC response (6), it is essential to remove DC from the responder cells. For example, using culture conditions and assay procedures described in Table I, PFC (± SEM) per culture were 25 ± 6, 55 ± 10, 145 ± 35, 465 ± 70, and 495 ± 75 for cultures to which either none, 1, 3, 10, or 30 × 10⁴ DC were added, respectively, to the responder cells. On the basis of this and other repeated titrations, we elected to use 10⁵ DC per culture of 10⁶ responder cells.

Taking advantage of the fact that our preparations of NK cells lose cytotoxicity in culture, DC, SRBC, and NK cells were coincubated for 24 h; then responding

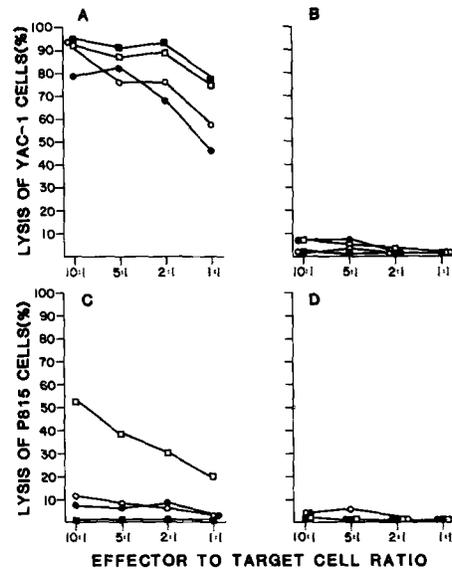


FIGURE 2. Cytotoxicity of NK cells sorted using the FACS and assayed either immediately or after 24 h in culture. NK cells were treated with anti-Ly-1.1, anti-Ly-2.1, anti-Ia, and C'. Cells were then stained with anti-Thy-1-FITC and sorted into Thy-1⁻ NK cells (■) and Thy-1⁺ NK cells (□). Controls included NK cells which were stained with anti-Thy-1-FITC but not sorted (●), and unstained NK cells which were not sorted (○). The four preparations of cells were assayed against either YAC-1 (A and B) or P815 (C and D) target cells either immediately (A and C) or 24 h later (B and D).

TABLE I
NK Cells Suppress the Plaque-forming Cell Response

Exp.	Cell additions				Responses on day 5 (PFC/culture)
	Day -1*		Day 0 [‡]		
	DC	NK	Responder cells	DC	
1	+	-	+	-	975 ± 90
	+	+	+	-	120 ± 20
	+	+	+	+	2,265 ± 155
	-	+	+	-	<100
	-	+	+	+	1,320 ± 125
	-	-	+	-	<100
2	+	-	+	-	518 ± 20
	+	+	+	-	27 ± 5
	+	+ [§]	+	-	60 ± 20

* Cultures contained 10⁵ DC and 10⁵ NK cells as indicated. All cultures contained 4 × 10⁶ SRBC.

[‡] 10⁶ responding lymphocytes were added along with 10⁵ DC where indicated.

[§] NK cells were treated with anti-Thy-1, anti-Ia, and C'.

lymphocytes were added with or without additional DC. As shown in Table I, Exp. 1, DC incubated with NK cells failed to support the PFC response; however, suppression caused by NK cells was reversed when additional DC were added along with the responding lymphocytes.

In other experiments the reverse was done, i.e., NK cells were coincubated with responding lymphocytes and SRBC for 24 h before DC were added to

TABLE II
Thy-1⁻ NK Cells but not Thy-1⁺ Cells Suppress DC Function

Cell additions			Responses [‡] (PFC/culture)		Cytotoxicity of NK cells added on day -1	
Day -1*		Day 0 [‡]	Exp. 1	Exp. 2	Exp. 1	Exp. 2
DC	NK	Responder cells				
+	None	+	325 ± 38	161 ± 18	—	—
+	Thy-1 ⁺	+	245 ± 25	231 ± 12	69	77
+	Thy-1 ⁻	+	30 ± 22	10 ± 7	53	80
+	Anti-Thy-1-FITC (not sorted)	+	10 ± 10	8 ± 6	45	56
+	Unstained (not sorted)	+	0	2 ± 3	30	51
None	None	+	0	5 ± 3	—	—
None	None	Whole spleen	375 ± 75	—	—	—

* Cultures contained 10⁵ DC and 10⁶ of different preparations of NK cells. All cultures contained 4 × 10⁶ SRBC. The cytotoxicity of the NK cells used in the two experiments is recorded in the right-hand two columns as the percent lysis of YAC-1 cells at an E/T ratio of 1:1.

[‡] 10⁶ responder cells or whole spleen cells were added as indicated.

[‡] In Exp. 1 responses were measured on day 5 and in Exp. 2 responses were measured on day 4.

cultures. The PFC per culture were 1,130 ± 180 with NK cells and 1,430 ± 210 without NK cells; cultures of lymphocytes, and SRBC without DC had <100 PFC/culture. Thus, responding lymphocytes were not targets for NK cells.

Thy-1⁻ but not Thy-1⁺ NK Cells Suppress DC. NK cells treated with anti-Thy-1, anti-Ia, and C' retained full capacity to suppress PFC responses (Table I, Exp. 2). To determine whether Thy-1⁺ NK cells also caused suppression, NK cells treated with anti-Ly-1.1, anti-Ly-2.1, anti-Ia, and C' were stained with anti-Thy-1-FITC. The sorted Thy-1⁺ and Thy-1⁻ NK cell populations, as well as the stained-unsorted and the unstained-unsorted populations, had high cytotoxic activity against YAC-1 (Table II). Each of the four different populations of NK cells were coincubated with DC and SRBC for 24 h. Responding lymphocytes were then added to cultures and PFC responses were assayed 5 or 4 d later, (Table II, Exps. 1 and 2, respectively). Thy-1⁻ but not Thy-1⁺ NK cells suppressed PFC responses; clearly the staining and sorting procedures had no significant affect either on the cytotoxicity of NK cells or on the capacity of Thy-1⁻ NK cells to suppress PFC responses. These results taken together with the findings presented in Table I and previously (1), indicate that only Thy-1⁻ NK cells suppress lymphocyte proliferation by suppressing or eliminating DC that have interacted with antigen.

Discussion

Both Thy-1⁺ and Thy-1⁻ NK cells share qualities that are generally attributed to NK cells; i.e., both populations are inducible, have the morphology of large granular lymphocytes, are cytotoxic for YAC-1 target cells, and express the AGM1 marker. The present studies do not resolve whether the populations represent phenotypically different clones or whether the different populations represent different phases of activation or maturation of a single cell lineage.

To our knowledge, all presently available cloned NK lines are Thy-1⁺ (7, 8). At least certain Thy-1⁺ NK lines are cytotoxic for activated B cells (9). Recent

studies indicate rearrangement of T cell receptor β -chain genes in these cloned Thy-1⁺ NK lines (10). It should now be possible to select and establish cloned Thy-1⁻ NK lines that would be expected to suppress both B and T cell-mediated immunity by their effect on DC. A comparison of such Thy-1⁻ and Thy-1⁺ lines should provide insight into the origins of the two types of NK cells and of the different ways these populations may regulate immunity.

Summary

Cells enriched for NK activity (poly I:C induced, x-ray resistant, and nonadherent), include two phenotypically and functionally different populations. Both populations of NK cells are AGM1⁺, Ly-1.1⁻, Ly-2.1⁻, Ia⁻, and have the morphology of large granular lymphocytes. One population, however, is Thy-1⁺ while the second population is Thy-1⁻. Thy-1⁺ NK cells lyse YAC-1 and P815 target cells; Thy-1⁻ NK cells lyse YAC-1 but not P815 target cells. The FACS was used to obtain homogeneous populations of Thy-1⁺ and Thy-1⁻ NK cells, which retain high cytotoxicity. While Thy-1⁻ NK cells suppress the antibody response in vitro by suppressing or eliminating DC, Thy-1⁺ NK cells do not suppress antibody responses in vitro.

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References

1. Shah, P. D., S. M. Gilbertson, and D. A. Rowley. 1985. Dendritic cells that have interacted with antigen are targets for natural killer cells. *J. Exp. Med.* 162:625.
2. Abruzzo, L. V., and D. A. Rowley. 1983. Homeostasis of the antibody response: immunoregulation by NK cells. *Science (Wash. DC)*. 222:581.
3. Steinman, R. M., and M. C. Nussenzweig. 1980. Dendritic cells: features and functions. *Immunol. Rev.* 53:127.
4. Nussenzweig, M. C., R. M. Steinman, M. D. Witmer, and B. Gutchinov. 1982. A monoclonal antibody-specific for mouse dendritic cells. *Proc. Natl. Acad. Sci. USA.* 79:161.
5. Blakeslee, D., and M. G. Baines. 1976. Immunofluorescence using dichlorotriazinylaminofluorescein (DTAF). I. Preparation and fractionation of labelled IgG. *J. Immunol. Methods.* 13:305.
6. Inaba, K., R. M. Steinman, W. C. Van Voorhis, and S. Muramatsu. 1983. Dendritic cells are critical accessory cells for thymus-dependent antibody responses in mouse and in man. *Proc. Natl. Acad. Sci. USA.* 80:6041.
7. Dennert, G., Y. Ganesa, and S. Yamagata. 1981. Cloned cell lines with natural killer activity: specificity, function and cell surface markers. *J. Exp. Med.* 153:545.
8. Nabel, G., L. R. Bucalo, J. Allard, H. Wigzell, and H. Cantor. 1981. Multiple activities of a cloned cell line mediating natural killer cell function. *J. Exp. Med.* 153:1582.
9. Nabel, G., J. W. Allard, and H. Cantor. 1982. A cloned cell line mediating natural killer cell function inhibits immunoglobulin secretion. *J. Exp. Med.* 156:658.
10. Yanagi, Y., N. Caccia, M. Kronenberg, B. Chin, J. Roder, D. Rohel, T. Kiyohara, R. Lauzon, B. Toyonaga, K. Rosenthal, G. Dennert, H. Acha-Orbea, H. Hengartner, L. Hood, and T. W. Mak. 1985. Gene rearrangement in cells with natural killer activity and expression of the β -chain of the T cell antigen receptor. *Nature (Lond.)*. 314:631.