

# CD69-mediated Pathway of Lymphocyte Activation: Anti-CD69 Monoclonal Antibodies Trigger the Cytolytic Activity of Different Lymphoid Effector Cells with the Exception of Cytolytic T Lymphocytes Expressing T Cell Receptor $\alpha/\beta$

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## Summary

The effect of anti-CD69 monoclonal antibodies (mAbs) on the induction of the cytolytic activity in different types of lymphoid effector cells has been investigated. Three anti-CD69 mAbs, including the reference mAb MLR3 and two new mAbs (c227 and 31C4), have been used. All cloned CD3<sup>-</sup>CD16<sup>+</sup> natural killer (NK) cells belonging to different subsets (as defined by the surface expression of GL183 and/or EB6 antigens) were efficiently triggered by anti-CD69 mAbs and lysed P815 mastocytoma cells in a redirected killing assay. Triggering of the cytolytic activity could also be induced in CD3<sup>-</sup>CD16<sup>-</sup> NK clones, which fail to respond to other stimuli (including anti-CD16, anti-CD2 mAbs, or phytohemagglutinin). A similar triggering effect was detected in T cell receptor (TCR)  $\gamma/\delta$ <sup>+</sup> clones belonging to different subsets. On the other hand, anti-CD69 mAbs could not induce triggering of the cytolytic activity in TCR  $\alpha/\beta$ <sup>+</sup> cytolytic clones. Since all thymocytes are known to express CD69 antigen after cell activation, we analyzed a series of phenotypically different cytolytic thymocyte populations and clones for their responsiveness to anti-CD69 mAb in a redirected killing assay. Again, anti-CD69 mAb triggered TCR  $\gamma/\delta$ <sup>+</sup> but not TCR  $\alpha/\beta$ <sup>+</sup> thymocytes. Anti-CD69 mAb efficiently triggered the cytolytic activity of "early" thymocytes lines or clones (CD3<sup>-</sup>4<sup>-</sup>8<sup>-</sup>7<sup>+</sup>), which lack all other known pathways of cell activation. Thus, it appears that CD69 molecules may initiate a pathway of activation of cytolytic functions common to a number of activated effector lymphocytes with the remarkable exception of TCR  $\alpha/\beta$ <sup>+</sup> cytolytic cells.

Triggering of the cytolytic machinery of human effector lymphocytes can be induced by different stimulatory agents including antigens, mitogens, and appropriate mAbs. For example, mAbs specific for the CD3/TCR complex (1, 2) or CD2 molecules (3–5) can mediate triggering of the cytolytic activity in T lymphocytes expressing either TCR  $\alpha/\beta$  or TCR  $\gamma/\delta$ . On the other hand, activation of CD3<sup>-</sup>CD16<sup>+</sup> NK cells can be induced by anti-CD16 (6–8) or anti-CD2 (6, 7) mAbs. More recently, a new family of stimulatory surface molecules (9, 10) has been identified in discrete NK subsets. mAbs to these molecules, including GL183 and EB6 mAbs, selectively triggered these NK subsets. In addition, a number of triggering molecules are expressed at the lymphocyte surface only after cell activation. Among these surface molecules, the CD69 antigen is expressed very early after T or NK cell activation (11–13), whereas it is not

expressed on resting T or NK lymphocytes (11, 14, 15). It has been shown that anti-CD69 mAbs mediate T lymphocyte triggering leading to lymphokine production and cell proliferation (13, 16). On the other hand, in conventional TCR  $\alpha/\beta$ <sup>+</sup> cytolytic clones, anti-CD69 mAbs failed to induce cytolytic activity in redirected killing assays (16).

In the present study, we show that mAbs to CD69 molecules efficiently trigger the cytolytic machinery of a number of peripheral blood or thymus-derived cytolytic clones with the remarkable exception of those expressing  $\alpha/\beta$  TCRs.

## Materials and Methods

*Isolation and Culture of Lymphocyte Subsets.* PBL from normal volunteers were isolated by Ficoll-Hypaque gradients and subsequently separated into different subsets by cell sorting and/or treat-

ment with mAbs followed by complement depletion (10, 17, 18). Cloning of the various cell subsets was performed under limiting dilution conditions in the presence of irradiated feeder cells and IL-2 (rIL-2; Cetus Corp., Emeryville, CA), as previously described for both T and NK cells (10, 17, 18).

**Production of mAb** 5-wk-old male BALB/c mice were immunized with a cell clone termed CES9 (surface phenotype: CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup>, as previously described (10). After six injections, the mice were splenectomized and immune splenocytes were fused with P3U1 myeloma cells. The screening of hybridoma supernatants was based on the ability to modulate the cytolytic activity of CES9 against the cell line termed P815 used as a source of <sup>51</sup>Cr-labeled target cells in a 4-h <sup>51</sup>Cr release assay. According to this screening procedure, a hybridoma, termed c227 (IgG1), which was able to increase the cytolytic activity of CES9 clone against P815 target cells, was isolated and further subcloned in limiting dilution. The 31C4 mAb (IgG2a) was obtained using a procedure similar to that described above. In this case, however, mice were immunized with a polyclonal thymocyte population expressing the CD3<sup>-</sup>4<sup>-</sup>8<sup>-</sup>7<sup>+</sup>16<sup>-</sup> surface phenotype.

**Isolation and Culture of Thymocyte Subsets.** Normal human thymocytes were obtained from thymus fragments removed during cardiac surgery of patients 2 mo to 4 yr old, as described (19). CD3<sup>+</sup>γ/δ<sup>+</sup> thymocytes (1–2% of total thymus) were obtained after positive selection of CD3<sup>+</sup> thymic cells using ox-red blood cells immunocoated with OKT3 (anti-CD3 mAb), as described (20), followed by negative depletion of CD4<sup>+</sup> and CD8<sup>+</sup> cells (19). The resulting cell population was >95% CD3<sup>+</sup> TCR γ/δ<sup>+</sup>, as assessed by immunofluorescence using anti-TCR δ1 mAb. Cloning of either TCR α/β or TCR γ/δ<sup>+</sup> thymic cells was performed under limiting dilution conditions as previously described (21). CD1<sup>-</sup>3<sup>-</sup>4<sup>-</sup>8<sup>-</sup>7<sup>+</sup> thymocytes were obtained after negative depletion by panning and magnetic beads using anti-CD1 (OKT6), anti-CD3 (Leu4), anti-CD4 (Leu3a), and anti-CD8 (Leu2a) mAbs as described (22). After this purification, cells recovered were >99.9% CD1<sup>-</sup>3<sup>-</sup>4<sup>-</sup>8<sup>-</sup>. In vitro expansion and cloning of CD1<sup>-</sup>3<sup>-</sup>4<sup>-</sup>8<sup>-</sup> thymic cells was obtained as described (23).

**Functional Analysis of Cytolytic Clones.** The cytolytic activity of the various clones was tested in a 4-h <sup>51</sup>Cr-release assay, as described above. In all instances, target cells were used at a concentration of 5 × 10<sup>4</sup>/ml and were represented by the P815 murine tumor cell line. The E/T ratios ranged from 10:1 to 0.5:1, as indicated. After titration, DEAE-Sephacel-purified c227 and 31C4 mAbs were used in most experiments at a concentration of 0.2 ng/ml resuspended in 50 μl of medium. The other mAbs, including MLR3 (anti-CD69 IgG2a) (24), Leu23 (anti-CD69 IgG1) (kindly provided by Dr. R. Testi, University of LAquila, Italy), c218 (anti-CD56 IgG1), c127 (anti-CD16 IgG1), KD1 (anti-CD16 IgG2a), JT3A (anti-CD3 IgG2a), MAR206 (anti-CD2 IgG1), BB3 (anti-TCR γ/δ IgG1), and A13 (anti-TCR γ/δ IgG1) were used at doses ranging from 2 to 0.2 ng/ml, depending upon preliminary titration experiments. The above-mentioned mAbs were added at the onset of the cytolytic test together with the effector and target cells.

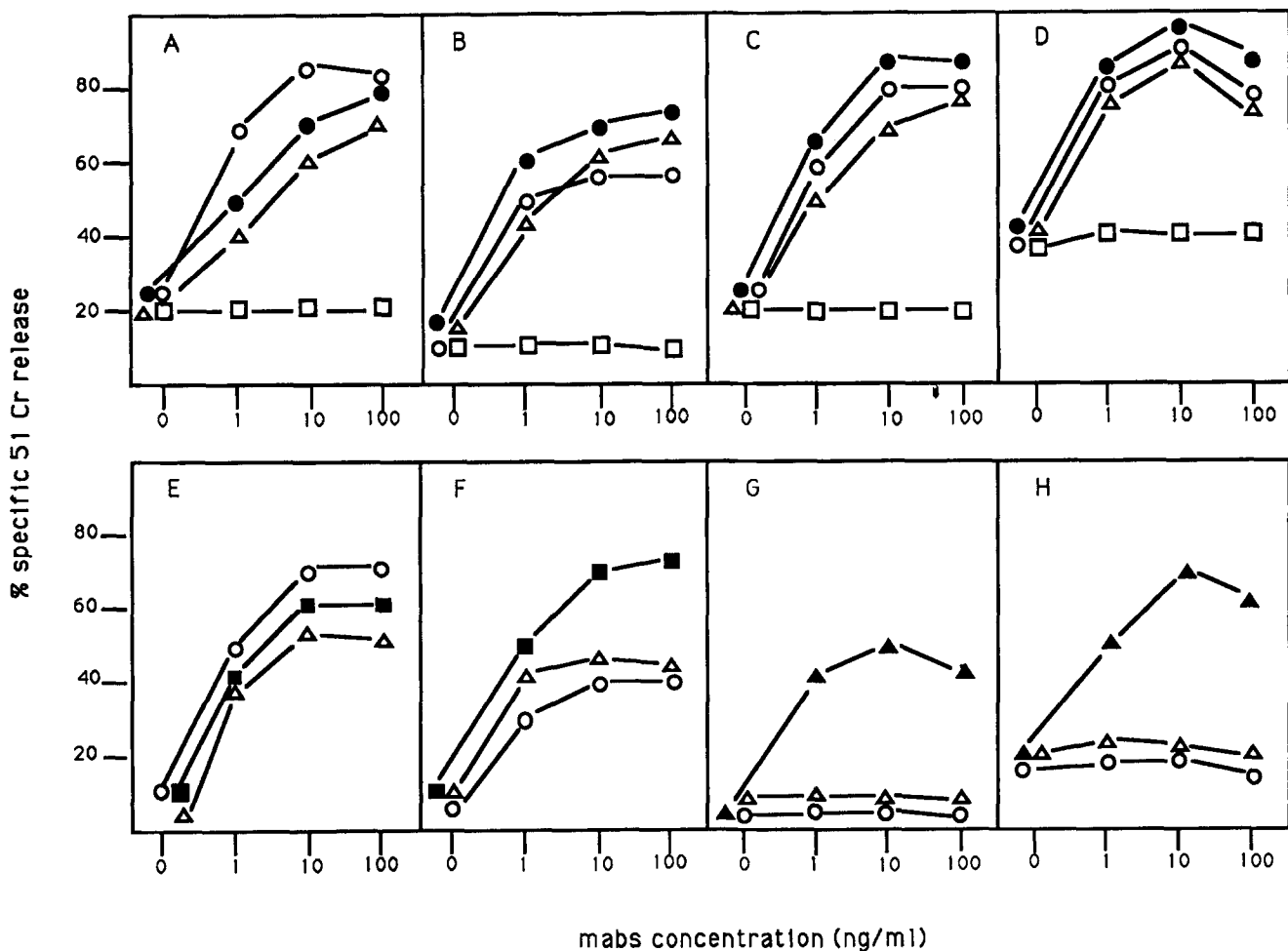
## Results and Discussion

In these studies we used three different anti-CD69 mAbs, including MLR3 (24) and two new mAbs, termed c227 and 31C4. The reactivity of c227 and 31C4 mAbs with CD69 molecules was substantiated by biochemical evidence and by the pattern of distribution of the corresponding antigen in resting and activated lymphocytes (not shown). Thus, under

reducing conditions, c227, 31C4, or the reference mAb MLR3 immunoprecipitated a 28–32-kD disulphide-linked dimer, which under nonreducing conditions migrated as a diffuse 60-kD band. In addition, analysis of the pattern of distribution in a panel of resting or activated peripheral blood-derived lymphocyte populations revealed that the c227- and the 31C4-defined antigen(s) were expressed only on activated lymphocyte populations and, in all instances, their distribution paralleled the expression of CD69 antigen (as defined by MLR3 mAb) (not shown).

**Anti-CD69 mAbs Trigger the Cytolytic Machinery of Different Lymphoid Cell Populations Derived from Peripheral Blood.** Previous studies (13, 16) indicated that mAbs specific for CD69 molecules mediated T cell triggering leading to intracellular Ca<sup>2+</sup> increases, and, in the presence of PMA, to lymphokine production and cell proliferation. However, no cytolytic activity in CD4<sup>+</sup> or CD8<sup>+</sup> cytolytic clones could be induced in redirected killing assays against P815 target cells using anti-CD69 mAbs (16). It should be noted that c227 and 31C4 mAbs had been originally selected on the basis of their ability to trigger the cytolytic activity of the immunizing cells (the NK clone CES9 or a CD3<sup>-</sup>4<sup>-</sup>8<sup>-</sup>7<sup>+</sup> thymocyte population) in redirected killing assays against P815 target cells. In view of these findings, we analyzed the ability of anti-CD69 mAbs to induce the cytolytic function in a panel of cytolytic lymphoid populations and clones, including NK cells, TCR α/β<sup>+</sup>, and TCR γ/δ<sup>+</sup> cells.

Recently, by the use of mAbs (GL183 and EB6) directed to novel triggering surface molecules selectively expressed by NK cell fractions, we could define four distinct NK subsets characterized by different functional properties (9, 10). We therefore investigated the effect of anti-CD69 mAbs in clones representative of the four subsets. As shown in Fig. 1, MLR3 and c227 mAbs enhanced the cytolytic activity not only of the immunizing clone CES9 (belonging to the GL183<sup>-</sup>EB6<sup>-</sup> subset) (D), but also triggered NK clones representative of the other phenotypic groups (A, B, and C). In all instances, the anti-CD69 mAb-induced cytolytic activity was comparable to that induced by KD1 mAb (anti-CD16). A similar triggering effect was elicited by other anti-CD69 mAbs, including 31C4 and Leu23 mAbs (not shown). Resting NK cells do not express CD69 constitutively (11, 14, 15), however, CD69 molecules are expressed after short-term culture in the presence of IL-2. We therefore investigated the time interval required for the acquisition of cytolytic responses to triggering with anti-CD69 mAb. In these experiments, CD3<sup>-</sup>56<sup>+</sup> lymphocyte populations were purified from peripheral blood and tested, at different intervals, for cytolytic responses to anti-CD69 mAbs (against P815 target cells). In a representative experiment, fresh NK cells lysed P815 cells in the presence of the (anti-CD16) KD1 mAb (53% lysis at 3:1 E/T ratio), but not of 31C4 (anti-CD69) or c218 (anti-CD56). After 18 h of culture, both anti-CD16 and anti-CD69 mAbs induced efficient target cell lysis (92% and 59%, respectively, at 3:1 E/T ratio); target cell lysis in the absence of mAb or in the presence of anti-CD56 mAb was 21% and 20%, respectively. After 36 h, the anti-CD69 and the anti-



**Figure 1.** Enhancements of cytolytic activities by c227 and MLR3 (anti-CD69) mAbs. (A-D) Phenotypically different CD3<sup>-</sup>CD16<sup>+</sup> clones tested for cytolytic activity against <sup>51</sup>Cr-labeled P815 target cells at an E/T cell ratio of 1:1. At the onset of the cytolytic test, graded amounts of mAb were added to the culture. (O) c227; (Δ) MLR3; (●) KD1 (anti-CD16); (□) c218 (anti-CD56). (A) Clone CA8.5 (GL183<sup>+</sup>, EB6<sup>-</sup>); (B) clone CEG76 (GL183<sup>+</sup>, EB6<sup>+</sup>); (C) clone A.M.25 (GL183<sup>-</sup>, EB6<sup>+</sup>); and (D) clone CES9 (GL183<sup>-</sup>, EB6<sup>-</sup>). (E-H) Phenotypically distinct CD3<sup>+</sup> clones tested for cytolytic activity against P815 cells at an E/T cell ratio of 5:1. At the onset of the cytolytic test were added graded amounts of either anti-TCR (BB3 or A13) mAb (■) or anti-CD3 (JT3A) mAb (▲), or anti-CD69 c227 (O) or MLR3 (Δ) mAb. (E) Clone D1.6 (TCR-γ/δ<sup>+</sup>, BB3<sup>+</sup>); (F) clone D1.12 (TCR γ/δ<sup>+</sup>, A13<sup>+</sup>); (G) clone 1B11 (TCR α/β<sup>+</sup>, CD8<sup>+</sup>); and (H) clone BG2 (TCR α/β<sup>+</sup>, CD8<sup>-</sup>, CD4<sup>-</sup>).

CD16 mAb-mediated lysis was 71% and 82%, respectively (22% and 19%, respectively, in the absence of mAb or in the presence of anti-CD56 mAb). These data are in line with a previous report by Lanier et al. (11) showing that full expression of CD69 molecules at the NK cell surface requires ~18 h of culture in the presence of rIL-2.

A minor subset of peripheral blood NK cells is characterized by the absence of surface CD16 antigens and by an increased expression of CD56 antigen (CD56 bright cells) (25). We analyzed clones derived from this cell subset for their responsiveness to anti-CD69 mAbs. These clones maintained the original surface phenotype and, similar to most CD16<sup>+</sup> clones, displayed a strong cytolytic activity against both NK-susceptible and NK-resistant target cells. In Table 1, two representative CD3<sup>-</sup>CD16<sup>-</sup>CD56<sup>+</sup> clones, termed E12 and AMK3, were compared to the CD16<sup>+</sup> clone CEG76 (10). As expected, clones E12 and AMK3 were unresponsive to

anti-CD16 mAb. In addition, they were poorly stimulated by anti-CD2 mAbs (not shown) or PHA. On the contrary, in these clones, anti-CD69 mAbs elicited a strong cytolytic activity.

We next analyzed a panel of T cell clones bearing either TCR α/β or TCR γ/δ (Fig. 1). The two TCR γ/δ<sup>+</sup> clones (E and F) are representative of the two major subsets of TCR γ/δ<sup>+</sup> lymphocytes present in peripheral blood (18). Thus, clone D.1.6 (E) reacted with BB3 mAb (26) and expressed Vδ2, whereas clone D.1.12 (F) was stained by A13 mAb and thus expressed Vδ1 (27). As shown in Fig. 1, anti-CD69 mAbs induced triggering of cytolytic activity in both clones. Similar results were obtained in eight additional TCR γ/δ<sup>+</sup> clones expressing either Vδ1 or Vδ2 (not shown).

Fig. 1, G and H, shows that anti-CD69 mAbs failed to trigger the cytolytic function of two representative TCR α/β<sup>+</sup> cytolytic clones. Clone 1B11 (G) is a typical CD4<sup>-</sup>

**Table 1.** Anti-CD69 mAbs Trigger the Cytolytic Function of CD3<sup>-</sup>CD16<sup>-</sup>CD56<sup>+</sup> NK Clones

Clone	Stimuli added to the cytolytic test*			
	None	Anti-CD16	Anti-CD69	PHA
E12 <sup>†</sup>	18 <sup>§</sup>	18	45	26
AMK3	19	18	57	28
CEG76	12	59	62	55

\* The mAbs used in this experiment were represented by KD1 (anti-CD16) and 31C4 (anti-CD69), used at a final concentration of 1 ng/ml. PHA was used at a final dilution of 1:1,000 (vol/vol).

<sup>†</sup> Effector cells were either CD3<sup>-</sup>16<sup>-</sup>56<sup>+</sup> clones (E12 and AMK3) or a conventional CD3<sup>-</sup>16<sup>+</sup>56<sup>+</sup> clone (CEG76). Target cells were represented by the P815 mastocytoma cell line.

<sup>§</sup> Data are expressed as a percent specific <sup>51</sup>Cr release at an E/T cell ratio of 1:1.

CD8<sup>+</sup> cytolytic clone, whereas clone BG2 (*H*) is an unusual CD4<sup>-</sup>CD8<sup>-</sup> TCR  $\alpha/\beta$ <sup>+</sup> clone also characterized by a strong non-MHC-restricted cytolytic activity against K562 target cells (not shown). That the TCR expressed by clone BG2 is indeed a TCR  $\alpha/\beta$  was indicated by the reactivity with the WT31 mAb and by the biochemical characteristics of immunoprecipitated TCR molecules. It should be noted that, although unresponsive to anti-CD69 mAbs, the cytolytic activity of both TCR  $\alpha/\beta$ <sup>+</sup> clones against P815 cells could be efficiently triggered by anti-CD3 mAbs.

**Triggering of Cytolytic Activity by Anti-CD69 mAbs in Thymocyte Populations and Clones.** As shown by previous studies, CD69 antigen is constitutively expressed on a subset of freshly isolated thymocytes that display bright CD3 fluorescence (28). Cells belonging to this subset were also characterized by the mutually exclusive expression of CD4 and CD8 antigens, a characteristic of the mature TCR  $\alpha/\beta$ <sup>+</sup> thymocyte populations. Anti-CD69 mAbs did not induce cytolytic activity of these thymocytes against P815 target cells. These data are not surprising since these cells belong to the TCR  $\alpha/\beta$ <sup>+</sup> subset (not shown). Also CD69<sup>-</sup> thymocytes were shown to express surface CD69 antigen after activation in culture (28). Therefore, we analyzed a series of thymocyte populations and clones expressing different surface phenotypes for their responses to anti-CD69 mAb. Since all of these cultured thymocytes expressed cytolytic activity (19, 23, 29), they could be analyzed in redirected killing assays (using P815 target cells) for their responsiveness to anti-CD69 mAbs. Similarly to clones derived from PB, thymocyte clones expressing TCR  $\alpha/\beta$  were unresponsive, whereas those expressing TCR  $\gamma/\delta$  were efficiently triggered by anti-CD69 mAbs (not shown).

We next analyzed CD3<sup>-</sup>4<sup>-</sup>8<sup>-</sup>7<sup>+</sup> thymocyte populations expanded in culture as previously described (23). These cells expressed cytoplasmic CD3 molecules, thus suggesting that they belong to an early stage of thymocyte differentiation

**Table 2.** Effect of mAbs on the Cytolytic Activity of Thymocyte Populations or Clones Bearing the CD3<sup>-</sup>4<sup>-</sup>8<sup>-</sup>7<sup>+</sup> Surface Phenotype

Effector cells	Stimuli added to the cytolytic test*					
	None	PHA	Anti-CD69			
			31C4	MLR3	Anti-CD3	Anti-CD16
Th41 <sup>†</sup>	20 <sup>§</sup>	45	56	50	21	21
Th50	26	56	50	ND	25	25
25A6	20	55	70	ND	21	20
10B9	4	70	60	55	3	2

\* The mAbs used in this experiment were represented by KD1 (anti-CD16), JT3A (anti-CD3), 31C4 (anti-CD69), and MLR3 (CD69), used at a final concentration of 1 ng/ml. PHA was used at a final dilution of 1:1,000 vol/vol.

<sup>†</sup> Effector cells were either bulk populations of activated thymocytes (Th41 and Th50) or thymocyte clones (25A6 and 10B9). In all instances these cells expressed the CD3<sup>-</sup>4<sup>-</sup>8<sup>-</sup>16<sup>-</sup>7<sup>+</sup> surface phenotype.

<sup>§</sup> Data are expressed as a percent specific <sup>51</sup>Cr release at an E/T ratio of 2:1.

(23). In addition to surface CD3, they lack other surface molecules involved in cell triggering including CD16 and CD2 molecules. Table 2 shows two polyclonal cell lines and two clones derived from this thymocyte population. It can be seen that anti-CD69 mAbs triggered a strong cytolytic response, comparable to that induced by PHA. As expected, neither anti-CD3 nor anti-CD16 mAbs could induce target cell lysis.

Taken together, our data provide evidence that CD69 antigens represent a triggering surface molecule in different types of cultured cytolytic lymphocytes, with the exception of TCR  $\alpha/\beta$ <sup>+</sup> cells. In a previous report, Testi et al. (16) could not detect induction of cytolytic activity by the anti-CD69 Leu23 mAb. However, these data are not in contrast with our present findings since the cytolytic cells used by these authors were TCR  $\alpha/\beta$ <sup>+</sup> clones.

Although not shown, triggering of cytolytic activity was also obtained using anti-CD69-producing hybridomas as "triggering targets". Thus, all types of cytolytic effector cells analyzed mediated lysis of the hybridoma target cells with the exception of TCR  $\alpha/\beta$ <sup>+</sup> cytolytic clones. These data indicate that triggering of cytolytic activity mediated by anti-CD69 mAbs (similar to most other known pathways of lymphocyte activation) can only be detected in experimental conditions that allow redirected killing (i.e., use of Fc $\gamma$ R-positive target cells, such as P815, or anti-CD69 mAb-producing hybridoma). On the contrary, anti-CD69 mAbs could not enhance or inhibit the lysis of Fc $\gamma$ R-negative target cells.

Interestingly, in two distinct CD3<sup>-</sup> cell populations also lacking CD16 surface antigen, cytolytic activity could be triggered only by anti-CD69 mAbs. Perhaps more importantly, in CD3<sup>-</sup>4<sup>-</sup>8<sup>-</sup>7<sup>+</sup> thymocyte lines, which also lack all of the known activation pathways functioning in T or NK cells,

CD69 represented the only surface molecule capable of inducing efficient cell triggering. These data may suggest a possible role of CD69 molecules in the regulation of the antigen-

independent proliferation occurring in immature thymocyte populations.

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