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Release of Esterase from Murine Lymphokine-activated Killer Cells in Antibody-dependent Cellular Cytotoxic Reaction

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Release of granule enzyme(s) (BLT esterase) in the antibody dependent lymphokine-activated killer (LAK) cell-mediated cytotoxic reaction (LAK ADCC) was studied using LAK cells induced from murine splenocytes and thymocytes, various human tumor cells and relevant monoclonal antibodies (mAbs) to the tumor cells. BLT esterase was not significantly released from LAK cells in direct LAK cell-mediated cytotoxic reactions (LAK CMC). However, cultures of LAK cells and IgG-coated target tumor cells resulted in release of the enzyme concomitantly with target cell lysis, although esterase release proceeded faster than target cell lysis. Anti-LFA-1 mAb showed an inhibitory effect on LAK CMC but not on either LAK ADCC or BLT esterase release in the ADCC. These results indicate that exocytosis of granule enzyme from LAK cells is triggered by stimulation of Fc receptor on LAK cells and that LAK CMC and LAK ADCC differ in their lytic mechanism in terms of the release of BLT esterase.

Key words: Antibody-dependent cellular cytotoxicity — Lymphokine-activated killer cell — Esterase — Fc receptor — Lymphocyte function-associated antigen 1

Lymphokine-activated killer (LAK[2]) cells display antibody-dependent cellular cytotoxicity (ADCC) in addition to their direct cytotoxicity against target tumor cells.[1,2] Although LAK cells induced from murine splenocytes contain cells that bear different cell surface phenotypes which classify them as NK and T cell type,[3,4] both types of LAK cells express Fc receptor as a common phenotype which enables LAK cells to act as the effector of ADCC.[1,2,5]

The cytotoxic mechanism of killer cells is indicated to be mediated by killer factors such as lymphotoxin (TNFβ), TNFα and pore-forming proteins.[6–10] Exocytosis of granules containing pore-forming proteins in cytotoxic T cells, NK cells or LAK cells has been proposed to be the critical event in the target cell lysis.[11,12] The cytotoxic granules of these killer cells also contain a family of serine esterases.[13–15] Therefore, such granule enzymes appear to be secreted concomitantly with the release of cytotoxic factors. For example, esterases are released from cytotoxic T cells by stimulation with target cells expressing the relevant antigen.[16] However, secretion of the granule enzymes in ADCC has not been determined, although their close association has been reported in the case of so-called reverse-type ADCC in which antibody bound to killer cells and Fc receptor-expressing target cells at the Fab and Fc portions, respectively.[17,18]

In this work, we investigated the secretion of granule enzyme(s) (BLT esterase) from LAK cells and demonstrated that it is triggered by the reaction between murine LAK cells and antibody-bound target cells, that the exocytosis of the esterase is mediated by stimulation of Fc receptor on LAK cells and that it is correlated with the ADCC activity of LAK cells.

MATERIALS AND METHODS

Animals Male C57BL/6 mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu and used at ages of 7–8 weeks.

Tumor cells Human cell lines, MOLT-4F acute lymphoblastic leukemia, Raji Burkitt lymphoma, K562 myelocytic leukemia, MKN-45 stomach cancer, SW1116 colon cancer, and MGH-U1 urinary bladder cancer cells, and mouse cell lines, YAC-1 T lymphoma and P815 mastocytoma, were used. These tumor cell lines were maintained by *in vitro* culture in RPMI 1640 medium containing penicillin G at 100 unit/ml, kanamycin sulfate at 60 μg/ml, 1 m*M* sodium pyruvate, 2 m*M* L-glutamine, HEPES at 2.3 mg/ml, $NaHCO_3$ at 2.0 mg/ml and 10% heat-inactivated fetal calf serum (FCS) (Flow Laboratories) in a CO_2 incubator. Monolayer culture cells (SW1116 and MGH-U1) were dispersed with phosphate-buffered saline (PBS) containing 0.1% ethylenediamine-

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[2] Abbreviations: LAK, lymphokine-activated killer; ADCC, antibody-dependent cellular cytotoxicity; CMC, cell-mediated cytotoxicity; LFA-1, lymphocyte function-associated antigen 1; BLT, N-α-benzyloxycarbonyl-L-lysine thiobenzyl ester.

tetraacetic acid by incubating them for a few minutes at 37°C.

Interleukin (IL) 2 Recombinant human IL 2 (rhIL-2) having a specific activity of 1.4×10^7 JU (Jurkat unit)/ml was kindly donated by Shionogi Pharmaceutical Co., Osaka, and used after appropriate dilution.

Preparation of LAK cells and the clones Aliquots (2.5×10^6 cells in 1 ml of RPMI 1640 containing 10% FCS) of fresh splenocytes from C57BL/6 mice were placed into wells of a Costar 12-well plastic plate and were incubated in a CO_2 incubator for 4 to 5 days at 37°C in the presence of rhIL-2 at 500 JU/ml. The cells collected from plates were washed with medium and used as LAK cells.

Antibodies Anti-mouse CD3 mAb, 145-2C11, was donated by Dr. K. Okumura, Medical School, Junten-do University, Tokyo. Mouse mAbs, HBJ127 (IgG_1), HBJ98 (IgG_{2a}), B3 (IgG_1) and a rat mAb, KBA (IgG_{2a}), were produced from the hybridomas which had been established in our laboratory.[19-22] Both HBJ127 and HBJ98 recognize a human cell proliferation-associated antigen (gp125) but epitopes recognized with these mAbs are different.[19] B3[20] and KBA[21, 22] recognize a rat homologue of human gp125[20] and mouse LFA-1, respectively. Ascites fluids containing high titer of HBJ127, HBJ98 or B3 were produced in BALB/c mice. For the preparation of KBA, the hybridoma cells were transplanted into BALB/c *nu/nu* mice that had been pretreated with an i.p. injection of Pristane (Aldrich Chemical Co., Milwaukee, Wis.). The ascites fluids were made up to 50% saturation with $(NH_4)_2SO_4$ and allowed to stand in the refrigerator for 4 h. The resultant precipitates were collected by centrifugation at 10000*g*, and dissolved in PBS. This solution was subjected to overnight dialysis against a large volume of PBS. The inner solution containing mAb was used for experiments.

Cytotoxicity assay The extent of target cell lysis was assessed by the ^{3}H-uridine method as reported previously.[23] Briefly, target cells were incubated overnight at 37°C in RPMI 1640 medium containing 10% FCS and ^{3}H-uridine (specific activity, 28 Ci/mmol; Amersham Japan, Tokyo) at 2 μCi/ml. The cells were washed 3 times with PBS and then adjusted to the concentration of 5×10^4 cells/ml. Various numbers of effector cells were mixed with 5×10^3 target cells in a final volume of 0.2 ml in wells of a Costar 96-well U-bottomed plastic plate, and the cell mixture was incubated for 18 h at 37°C. For ADCC assays, appropriately diluted mAb (10 μl/well) was also added. After incubation, cells were collected by the use of a Skatron cell harvester, and the radioactivity in the cells was measured by a standard liquid scintillation technique. The per cent target cell lysis was calculated from the equation: % Cytotoxicity = [1 − (cpm of target cells cultured with effector cells/cpm of target cells cultured alone)] × 100.

Assays for BLT esterase activity Esterase activity was assayed by the use of N-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) as a substrate. LAK cells were mixed with various numbers of target cells and mAbs. Aliquots (5×10^3 LAK cells in 0.2 ml) of the mixture were distributed in wells of a Costar 96-well U-bottomed plastic plate and cultured for 6 h at 37°C. After incubation, 20 μl aliquots of the culture supernatants were mixed with 180 μl of BLT solution containing 0.2 m*M* BLT, 0.22 m*M* 5,5′-dithio-bis(2-nitrobenzoic acid) in 0.2 *M* Tris-HCl buffer, pH 8.1. The mixture was incubated at room temperature for 30 min, and the absorbance of the solution at 414 nm was measured by the use of an Immunoreader NJ-2000 (Nippon InterMed, Japan). The percentage of BLT esterase release was calculated from the equation: % BLT esterase release = $100\times(E-S)/(T-S)$, where E and S represent the enzyme units in an experimental well with LAK cells and a control well without LAK cells, respectively; T represents total units of BLT esterase in LAK cells in a well.

RESULTS AND DISCUSSION

We studied first the cytotoxic activity of murine LAK cells against various human and murine tumor cells in the presence or absence of HBJ127 mAb (IgG_1), which recognizes a proliferation-associated glycoprotein of Mr 12500 (gp125) on human tumor cells.[19] The results are shown in Table I and Fig. 1. The cytotoxic activity of LAK cells to MOLT-4 human T lymphoma cells was augmented by pretreatment of the target cells with HBJ127 mAb reactive with these target tumor cells. MKN-45 human gastric cancer cells resistant to LAK cell-mediated cytotoxicity (CMC) were also destroyed by LAK cells in the presence of HBJ127. However, similar treatment of mouse tumor cells (YAC-1 and P815), which were unreactive with HBJ127, did not increase their LAK sensitivity (data not shown). Moreover, $F(ab')_2$ fragments of HBJ127 could not enhance the LAK activity, but they showed the activity in the presence of rabbit anti-mouse IgG. All these findings indicated that augmentation of LAK activity by addition of mAbs is caused by ADCC. As for isotypes of mAbs effective in ADCC, LAK cell-mediated ADCC took place with the help of mAbs of any isotype, as opposed to macrophage-mediated ADCC which is supported by IgG_{2a} and IgG_3 antibodies but not by IgG_{2b} or IgG_1 antibodies.[24, 25] This is probably due to different structures of Fc receptors present on macrophages and lymphocytes.

As to the LAK cell population responsible for BLT esterase release, we treated spleen cells with anti-CD3 antibody, 145-2C11 (for T cell depletion) or anti-asialoGM1 antibody (for NK cell depletion) and com-

Table I. Effect of Anti-LFA-1 mAb (KBA) on ADCC Activity of LAK Cells

Target cells	mAb[b]	% Cytotoxicity[a] (E/T=12)			
		–	HBJ127	KBA	KBA+HBJ127
MOLT-4		53.5±3.3[c]	81.9±1.5	8.4±3.1	71.6±7.3
Raji		26.9±4.0	44.5±1.0	6.1±2.0	21.9±1.2
MGH-U1		18.9±1.4	32.2±5.0	−2.6±9.9	37.5±3.8
P815		44.1±0.8	42.5±1.0	24.0±2.1	29.5±3.2
YAC-1		44.8±1.3	42.0±1.8	34.2±2.2	32.1±1.5

a) Cytotoxicity assay was performed by the ^{3}H-uridine method for 16 h (see "Materials and Methods").
b) The final concentration of HBJ127 mAb was 10 μg/ml and that of KBA mAb was 25 μg/ml.
c) Mean±SE of triplicates, expressed as percent cytotoxicity.

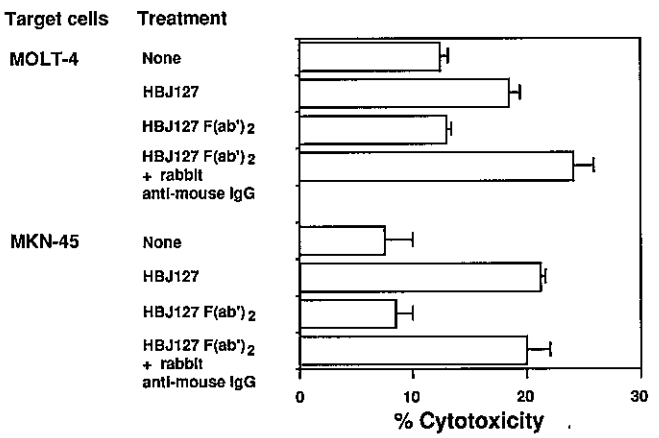

Fig. 1. LAK ADCC to human tumor cells in the presence of HBJ127 mAb or its $F(ab')_2$. LAK cells and target tumor cells were cultured for 18 h in the presence or absence of HBJ127 mAb or its $F(ab')_2$ and target cell lysis was determined as described in "Materials and Methods." Columns and bars show the means and SEM of triplicate wells, respectively.

plement and then cultured them in the presence of IL-2 for 4 days. Together with LAK cells induced from untreated spleen cells, these antibody-treated cells were tested for release of BLT esterase in the ADCC reaction using MOLT-4 cells and HBJ127 mAb. The contents of BLT esterase in the LAK cells from T cell-depleted (NK LAK) and from NK cell-depleted spleen cells (T LAK) were 3.3-fold and 0.4-fold of that in the LAK cells from untreated spleen cells, respectively. The released BLT esterase in ADCC was 9.3% from the NK LAK, 2.4%, from the T LAK and 6.3% from the spleen LAK. These findings indicated that BLT esterase release from the spleen cell LAK in ADCC is mainly attributable to NK-type LAK cells.

To study the different characters of the direct cytotoxic reaction of LAK cells against target cells (LAK

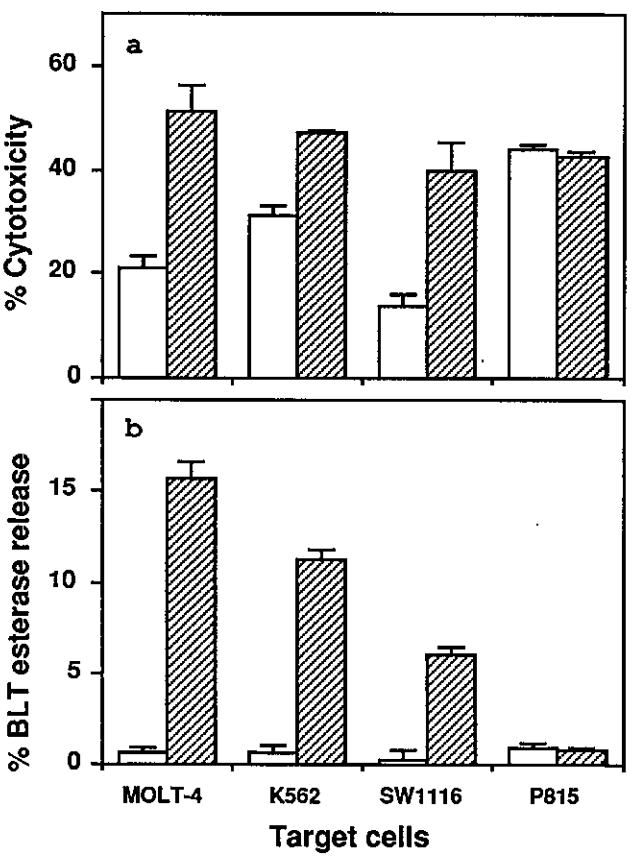

Fig. 2. Relation between target cell lysis and BLT esterase release from LAK cells. LAK cells and target tumor cells were cultured in the presence (▨) or absence of HBJ127 mAb (□) and lysis of target cells and release of BLT esterase were determined as described in "Materials and Methods." a, Cytotoxicity of LAK cells against human (MOLT-4, K562 and SW1116) and murine tumor cells (P815). E/T ratio=10. b, BLT esterase secretion from LAK cells cultured with the indicated target cells in the presence or absence of HBJ127. Columns and bars show the means and SEM of triplicate wells, respectively.

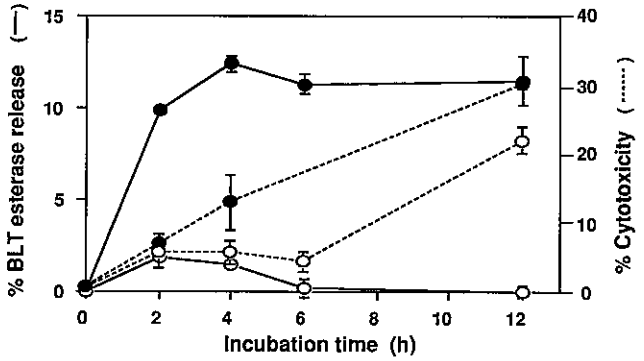

Fig. 3. Time course of ADCC activity of, and release of BLT esterase from, LAK cells. LAK cells were cultured with MOLT-4 cells in the presence (●) or absence (○) of HBJ127 and the activity of BLT esterase (—) and cytotoxic activity (···) were measured as described in "Materials and Methods." Circles and vertical bars show the means and SEM of triplicate wells, respectively.

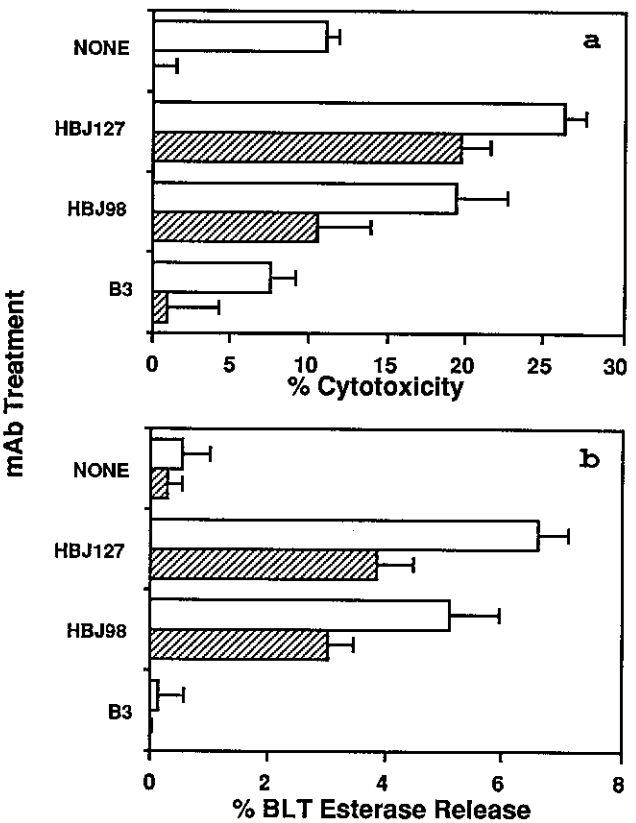

Fig. 4. Effect of an anti-LFA-1 mAb against cytolytic activity of LAK cells and BLT esterase release from LAK cells in ADCC. LAK cell activity (a) against MOLT-4 cells with the indicated mAbs (10 μg/ml) at the E/T ratio of 5 and BLT esterase release (b) from LAK cells at the E/T ratio of 1 were assayed in the absence (□) or presence of an anti-LFA-1 mAb, KBA (25 μg/ml) (▨) as described in "Materials and Methods." Columns and bars show the means and SEM of triplicate wells, respectively.

CMC) and the LAK ADCC, we examined the BLT esterase release from LAK cells. BLT esterases (serine esterase and granzymes A and B) are present at high levels in CTL, NK and LAK cells.[15, 16, 26] It is known that BLT esterase is secreted from CTL into the supernatant,[16] but not from LAK cells,[27] after their binding with target cells. The relationship between ADCC activity of LAK cells and release of BLT esterase is shown in Fig. 2. In the absence of mAb, BLT esterase was not found in the culture supernatants of LAK cells and target human tumor cells (MOLT-4, K562 and SW1116), regardless of the lysis of tumor cells (Fig. 2a). However, addition of HBJ127 mAb significantly increased the release of BLT esterase in the culture (Fig. 2b). A similar result was obtained by the use of MKN-45 tumor cells as a target. The antibody-target cell combinations negative for ADCC such as the combination of HBJ127 mAb and P815 mouse mastocytoma cells and combinations of B3 IgG_1 (anti-rat gp125 mAb) and human tumor cells did not result in release of BLT esterase from LAK cells. BLT esterase activity was not detected from the target tumor cells used in the experiments. These findings suggest an essential role of Fc receptor in the release of BLT esterase from LAK cells.

We next examined the dependence of BLT esterase release on the time of LAK ADCC using a combination of MOLT-4 cells and HBJ127 mAb. The result is depicted in Fig. 3. BLT esterase release and LAK ADCC showed different time courses. The secretion of BLT esterase from LAK cells induced by co-culture with HBJ127-sensitized MOLT-4 cells was faster than the advance of cell lysis, and it reached a plateau after 4 to 6 h when the target cell lysis was still increasing. This finding suggests that the BLT esterase release or granule exocytosis from LAK cells takes place in advance of target cell lysis by LAK ADCC.

To study further whether the secretion of BLT esterase is correlated directly with LAK ADCC, the BLT esterase release was determined in the presence of an anti-LFA-1 mAb, KBA, which could block LAK CMC by inhibiting the contact between LAK cells and target cells.[21] The results are shown in Fig. 4. To study whether LFA-1 is involved in LAK ADCC, we first examined the effect of KBA on LAK ADCC. The LAK activity against human tumor cells was decreased to less than 10% of the control by the addition of KBA. However, when HBJ127 was present, the LAK activity against MOLT-4, Raji and

MGH-U1 was displayed even in the presence of KBA (Table I). Other mAbs of IgG_{2a}, IgG_{2b} or IgG_3 isotype, which are reactive with the target cells, also exhibited ADCC activity in the presence of KBA (data not shown). We confirmed that KBA mAb reacted only to murine LAK cells but not to either target human tumor cells or HBJ127 mAb. When LAK cells were incubated with MOLT-4 cells, the LAK activity was enhanced by the addition of HBJ127 or HBJ98 with or without KBA. BLT esterase was detected in the supernatant of LAK cells incubated with MOLT-4 at E/T ratio of 1. Only target cells bound with HBJ127 or HBJ98 induced secretion of this granule enzyme from LAK cells, and similar results were obtained in the presence of KBA (Fig. 4b). These findings indicate that LFA-1 plays a leading role in LAK CMC but not in LAK ADCC and suggest that the LAK CMC and the LAK ADCC proceed through different processes of cytotoxicity.

As demonstrated above, in the regular ADCC, the target cells and effector cells adhere by binding with the Fab part and Fc part of an antibody, respectively. In addition to such regular ADCC, there is another type of ADCC called reverse-type ADCC, where Fab binds to killer cells and Fc to target cells. To study the relationship between the reverse-type ADCC and BLT esterase release, we examined the secretion of the esterase from LAK cells stimulated with anti-CD3ε mAb, 145-2C11, using P815 cells bearing Fc receptor as a target. In this reverse-type ADCC too, BLT release occurred concomitantly with the ADCC (19.4% at 6 h).

In either the regular LAK ADCC or the reverse ADCC, BLT esterase could be released from LAK cells but not from target cells, because BLT esterase activity was detected only from LAK cells but not from the target cells used. It has been reported that the stimulation of killer T cells with anti-CD3 mAb resulted in release of BLT esterase from the LAK cells.[18] However, in the regular LAK ADCC, the antibody binds to Fc receptor of LAK cells. Therefore, in the regular ADCC, the stimulation of Fc receptor may trigger the release of BLT esterase. To confirm this, we examined the effect of anti-Fc receptor substances on the release of BLT esterase in LAK ADCC. As expected, addition of anti-mouse Fc receptor mAb, 24G2 (25 μg/ml) and Protein A (10 μg/ml) to the LAK ADCC reaction inhibited BLT esterase release at 53% and 42%, respectively.

It has been proposed that the interaction between Fc receptor on killer cells and IgG-coated target cells not only mediates their conjugate formation but also activates killer cells. For example, increase of intracellular calcium ion concentration, stimulation of phagocytosis and production of reactive oxygen species could be induced by immune complex in macrophages and granulocytes.[28-30] On the other hand, stimulation of Fc receptors results in induction of calcium flux, enhancement of cytolytic activity and lymphokine production from NK cells and K cells.[31-33] These experimental findings supported the view that Fc receptor might act as a molecule for signal transduction. In this report, we have revealed an essential role of Fc receptors in signal transduction for release of granular enzyme from LAK cells. However, it remains uncertain whether the esterase itself is a killing factor in the ADCC reaction or whether some other killing factor(s) released concomitantly with the esterase actually mediates the target cell lysis. Moreover, LAK cells dealt with in the present experiments were a mixed cell population, as reported previously.[3] Therefore, it may be questionable whether the population responsible for ADCC and release of BLT esterase is the real LAK cell population or whether it is a population of third-party cells bearing Fc receptor. The findings that, at least in the unstimulated state, the ADCC activity of spleen cells was negligible (< 5% cytotoxicity to various target cells and mAbs) and that LAK cell clones bearing Fc receptor also showed a certain degree of ADCC activity (data will be reported elsewhere) would indicate that at least a part of IL 2-stimulated lymphocytes could show both direct cytotoxic activity and ADCC which leads to BLT esterase release.

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REFERENCES

1) Shiloni, E., Eisenthal, A., Sachs, D. and Rosenberg, S. A. Antibody-dependent cellular cytotoxicity mediated by murine lymphocytes activated in recombinant interleukin-2. *J. Immunol.*, **138**, 1992–1998 (1987).
2) Munn, D. H. and Cheung, N. V. Interleukin-2 enhancement of monoclonal antibody-mediated cellular cytotoxicity against human melanoma. *Cancer Res.*, **47**, 6600–6605 (1987).
3) Hashimoto, Y. and Nishimura, T. Phenotypes of lymphokine-activated killer (LAK) cells derived from various mouse lymphoid cell sources. *Gann Monogr. Cancer Res.*, **34**, 111–120 (1988).

4) Ortaldo, J. R., Mason, A. and Overton, R. Lymphokine-activated killer cells: analysis of progenitors and effectors. *J. Exp. Med.*, **164**, 1193–1205 (1986).
5) Eisenthal, A., Shiloni, E. and Rosenberg, S. A. Characterization of IL-2-induced murine cells which exhibit ADCC activity. *Cell. Immunol.*, **115**, 257–272 (1988).
6) Ortaldo, J. R., Mason, L. H., Mathieson, B. J., Liang, S., Flick, D. A. and Herberman, R. B. Mediation of mouse natural cytotoxic activity by tumor necrosis factor. *Nature*, **321**, 700–702 (1986).
7) Yamamoto, R. S., Ware, C. F. and Granger, G. A. The human LT system. XI. Identification of LT and "TNF-like" LT forms from stimulated natural killers, specific and nonspecific cytotoxic human T cells *in vitro*. *J. Immunol.*, **137**, 1878–1884 (1986).
8) Masson, D. and Tschopp, J. Isolation of a lytic, pore-forming protein (perforin) from cytolytic T-lymphocytes. *J. Biol. Chem.*, **260**, 9069–9072 (1985).
9) Young, J. D., Podack, E. R. and Cohn, Z. A. Properties of a purified pore-forming protein (perforin 1) isolated from H-2-restricted cytotoxic T cell granules. *J. Exp. Med.*, **164**, 144–155 (1986).
10) Liu, C., Perussia, B., Cohn, Z. A. and Young, J. D. Identification and characterization of a pore-forming protein of human peripheral blood natural killer cells. *J. Exp. Med.*, **164**, 2061–2076 (1986).
11) Podack, E. R., Young, J. D. and Cohn, Z. A. Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. *Proc. Natl. Acad. Sci. USA*, **82**, 8629–8633 (1985).
12) Zalman, L. S., Brothers, M. A., Chiu, F. J. and Müller-Eberhard, H. J. Mechanism of cytotoxicity of human large granular lymphocytes: relationship of the cytotoxic lymphocyte protein to the ninth component (C9) of human complement. *Proc. Natl. Acad. Sci. USA*, **83**, 5262–5266 (1986).
13) Pasternack, M., Verret, C. R., Liu, M. A. and Eisen, H. N. Serine esterase in cytolytic T lymphocyte. *Nature*, **322**, 740–743 (1986).
14) Masson, D., Nabholz, M., Estrade, C. and Tschopp, J. Granules of cytolytic T-lymphocytes contain two serine esterases. *EMBO J.*, **5**, 1595–1600 (1986).
15) Velotti, F., Palmieri, G., Morrone, S., Piccoli, M., Frati, L. and Santoni, A. Granzyme A expression by normal rat natural killer (NK) cells *in vivo* and by interleukin 2-activated NK cells *in vitro*. *Eur. J. Immunol.*, **19**, 575–578 (1989).
16) Takayama, H. and Sitkovsky, M. V. Antigen receptor-regulated exocytosis in cytotoxic T lymphocytes. *J. Exp. Med.*, **166**, 725–743 (1987).
17) Leo, O., Sachs, D. H., Samelson, L. E., Foo, M., Quinones, R., Gress, R. and Bluestone, J. A. Identification of monoclonal antibodies specific for the T cell receptor complex by Fc receptor-mediated CTL lysis. *J. Immunol.*, **137**, 3874–3880 (1986).
18) Takayama, H., Trenn, G., Humphrey, W., Jr., Bluestone, J. A., Henkart, P. A. and Sitkovsky, M. V. Antigen receptor-triggered secretion of a trypsin-type esterase from cytotoxic T lymphocytes. *J. Immunol.*, **138**, 566–569 (1986).
19) Masuko, T., Abe, J., Yagita, H. and Hashimoto, Y. Human bladder cancer cell-surface antigens recognized by murine monoclonal antibodies raised against T24 bladder cancer cells. *Jpn. J. Cancer Res.*, **76**, 386–394 (1985).
20) Hashimoto, Y., Masuko, T., Yagita, H., Endho, N., Kanazawa, J. and Tazawa, J. A proliferation-associated rat cell surface antigen recognized by a murine monoclonal antibody. *Gann*, **74**, 818–821 (1983).
21) Nishimura, T., Yagi, H., Yagita, H., Uchiyama, Y. and Hashimoto, Y. Lymphokine-activated cell-associated antigen involved in broad-reactive killer cell-mediated cytotoxicity. *Cell. Immunol.*, **94**, 122–132 (1985).
22) Nishimura, T., Yagi, H. and Hashimoto, Y. The role of lymphokine-activated cell-associated antigen. II. Distribution and correlation with cell cycle. *Cell. Immunol.*, **107**, 24–31 (1987).
23) Nishimura, T., Uchiyama, Y., Yagi, H. and Hashimoto, Y. Use of millipore diffusion chambers to assay *in vivo* IL-2 activity. *J. Immunol. Methods*, **78**, 239–245 (1985).
24) Johnson, W. J., Steplewski, Z., Matthews, T. J., Hamilton, T. A., Koprowski, H. and Adams, D. O. Cytolytic interactions between macrophages, tumor cells, and monoclonal antibodies: characterization of lytic conditions and requirements for effector activation. *J. Immunol.*, **136**, 4704–4713 (1986).
25) Hellström, I., Garrigues, U., Lavie, E. and Hellström, K. E. Antibody-mediated killing of human tumor cells by attached effector cells. *Cancer Res.*, **48**, 624–627 (1988).
26) Garcia-Sanz, J. A., Plaetinck, G., Velotti, F., Masson, D., Tschopp, J., MacDonald, H. R. and Nabholz, M. Perforin is present only in normal activated $Lyt2^+$ T lymphocytes and not in $L3T4^+$ cells, but the serine protease granzyme A is made by both subsets. *EMBO J.*, **6**, 933–938 (1987).
27) Zanovello, P., Rosato, A., Bronte, V., Cerundolo, V., Treves, S., Virgilio, F. D., Pozzan, T., Biasi, G. and Collavo, D. Interaction of lymphokine-activated killer cells with susceptible targets does not induce second messenger generation and cytolytic granule exocytosis. *J. Exp. Med.*, **170**, 665–677 (1989).
28) Young, J. D., Ko, S. S. and Cohn, Z. A. The increase in intracellular free calcium associated with IgGγ2b/γ1 Fc receptor-ligand interaction: role in phagocytes. *Proc. Natl. Acad. Sci. USA*, **81**, 5430–5434 (1984).
29) Di Virgilio, F., Meyer, B. C., Greenberg, S. and Silverstein, S. C. Fc receptor-mediated phagocytosis occurs in macrophages at exceedingly low cytosolic Ca^{2+} levels. *J. Cell Biol.*, **106**, 657–666 (1988).
30) Anderson, C. L., Guyre, P. M., Whitin, J. C., Ryan, D. H., Looney, R. J. and Fanger, M. W. Monoclonal antibodies to Fc receptors for IgG on human mononuclear phagocytes: antibody characterization and induction of superoxide production in a monocyte cell line. *J. Biol. Chem.*,

261, 12856–12864 (1986).

31) Cassatella, M. A., Anegon, I., Cuturi, M. C., Griskey, P., Trinchieri, G. and Perussia, B. FcγR (CD16) interaction with ligand induces Ca^{2+} mobilization and phosphoinositide turnover in human natural killer cells: role of Ca^{2+} in FcγR (CD16)-induced transcription and expression of lymphokine gene. *J. Exp. Med.*, **169,** 549–567 (1989).

32) Anasetti, C., Martin, P. J., Jure, C. H., Hellström, K. E., Ledbetter, J. A., Rabinovitch, P. S., Morishita, Y., Hellström, I. and Hansen, J. A. Induction of calcium flux and enhancement of cytolytic activity in natural killer cells by cross-linking of the sheep erythrocyte binding protein (CD2) and the Fc-receptor (CD16). *J. Immunol.*, **139,** 1772–1779 (1987).

33) Anegon, I., Cuturi, M. C., Trinchieri, G. and Perussia, B. Interaction of Fc receptor (CD16) ligands induces transcription of interleukin 2 receptor (CD25) and lymphokine genes and expression of their products in human natural killer cells. *J. Exp. Med.*, **167,** 452–472 (1988).