Brief Report [*]

Macrophage precursors derived from murine bone marrow develop into B220$^+$ LAK cells under the influence of Interleukin-2

A resemblance of these LAK cells to the reported NK-1.1$^+$ CD3$^-$ B220$^+$ LAK subset found in spleen cultures

(Short Title: Development of macrophage precursors into B220$^+$ LAK effectors)

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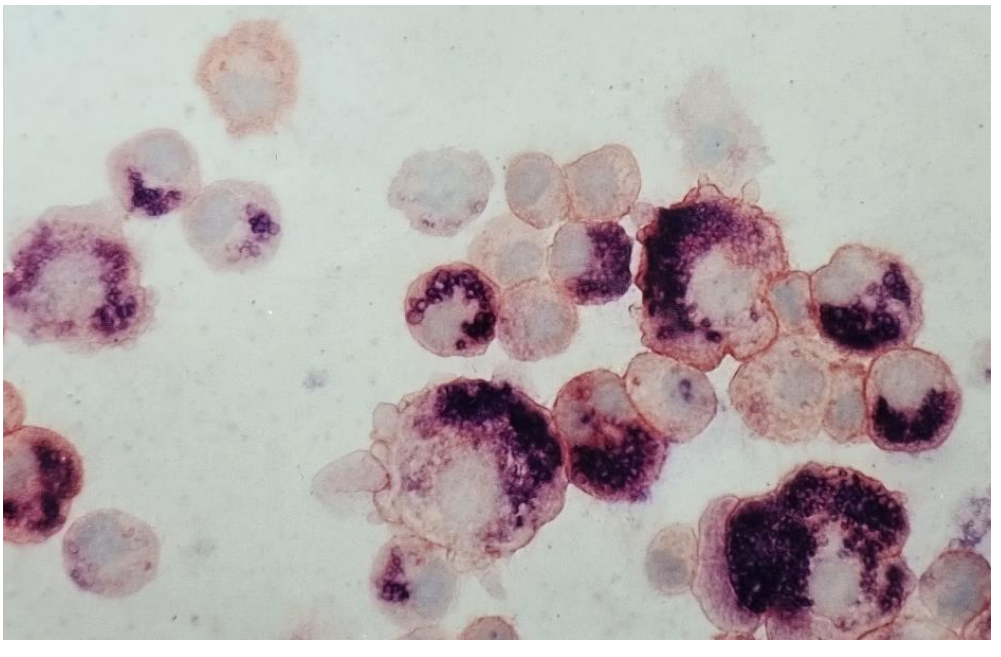
Key words: *macrophage precursor cell · interleukin-2 · B220 marker · NK cell lineage*



Immunohistochemical double staining for B220 (stained as brown) and cytosol protein perforin (stained as blue). The cells were obtained from 4-day LAK-culture.

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Abstract

Macrophage precursor cells obtained from murine bone marrow culture supplemented with interleukin-2 (IL-2) are known to possess NK/LAK-like characteristics. In the present work, these cells were tested for their expression of B220 marker on their surface, because the B220 marker was reported as a marker expressed on the activated stages of splenic NK cells. We found that, at the stage with highly lytic activity (LAK-activity), the effector cells derived from bone marrow macrophage precursors do express B220 marker on their surface. This kind of B220 acquisition on the macrophage precursor cells was induced by high-dose interleukin-2, but did not seem to be related with the production of cytosol protein perforin, a cytolytic molecule that can be induced in macrophage precursors upon the stimulation with interleukin-2. On the other hand, a down-regulation of the myeloid markers Mac-1 and F4/80 was observed on the surface of macrophage precursors when the cells began to express B220 marker. As a result, a highly lytic $B220^{+}$ population lacking myeloid cell markers appeared. Since this population displayed an antigenic profile ($B220^{+}$ NK-1.1^{+} $CD3^{-}$ $CD8^{-}$ Thy-1.2^{+}) that is similar to the LAK effectors derived from splenic NK cells, we suggest that this in vitro observed phenotypic variation of bone marrow macrophage precursors during their differentiation under the influence of interleukin-2 may represent the actual developing process of NK/LAK cells, of which the cell lineage has long been remained as unclarified.

Abbreviations used in this paper

BM	bone marrow
CSF- 1	colony-stimulating factor 1 (macrophage colony-stimulating factor)
GM-CSF	granulocyte-macrophage colony-stimulating factor
hr	human recombinant
IL-	interleukin-
LAK	lymphokine-activated killer
mAb	monoclonal antibody
mr	murine recombinant
M-CSF	macrophage colony-stimulating factor
MP	macrophage precursor
NK	natural killer
PBS	phosphate-buffered saline
TBS	Tris-buffered saline

INTRODUCTION

It is well known that the major part of LAK precursors in murine spleen can be divided into two mutually exclusive subsets: NK-like (NK-1.1$^+$ CD3$^-$ CD8$^-$) and T-like (NK-1.1$^-$ CD3$^+$ CD8$^+$) [1-4]. Under the stimulation with IL-2 or IL-4, both precursor populations develop into LAK effector cells, whereas their phenotypes regarding to the expression of NK-1.1 and T cell markers CD3, CD8 remain unchanged [2, 4]. However, the active lytic LAK effectors, either derived from NK-like or from T-like precursors, express B220 marker on their surface, although both precursor populations are completely negative for B220 [2, 4]. This kind of B220 expression on the LAK cells seems to be induced by the cytokines that induce LAK-activity and only the B220$^+$ cells in LAK-cultures display actively lytic activities [2, 4]. Therefore, the antigenic profiles of the two major populations of LAK cells should be more precisely defined as NK-1.1$^+$ CD3$^-$ CD8$^-$ B220$^+$ (from NK-like precursors) and NK-1.1$^-$ CD3$^+$ CD8$^+$ B220$^+$ (from T-like precursors). It was suggested that B220 expression may serve as a marker for cytokine-activated MHC-non-restricted killer cells [4].

We have established a method to efficiently generate LAK cells under the murine bone marrow culture conditions [5, 6]. The precursors for these bone marrow derived LAK cells were identified as macrophage precursors because they could quickly develop into macrophages under proper culture conditions [6]. Our observation of the relationship between NK-like cells and macrophages at bone marrow precursor level seems to be very interesting, as the lineage of NK cells has not yet been clarified although much of research work has been done in recent years [7-12]. Our experiments revealed that at very early differentiation stages, if IL-2 is present, the bone marrow macrophage precursors can develop into the cells with NK- and further with LAK-activities. The cells not only show morphological, phenotypic, and functional similarities to the splenic NK cells but also produce perforin as their lytic molecules [6]. In the present work, a further investigation on the IL-2-activated bone marrow macrophage precursors with NK/LAK characteristics was done, it was mainly focused on the study of the expression of B220 marker on these cells. Meanwhile, the mature macrophages derived from the same bone-marrow precursor population were also examined.

MATERIALS AND METHODS

Mice

Six to eight-week-old inbred C57BL6 (H-2b) mice were obtained from the Zentrale Versuchstieranstalt, Hannover, and were maintained in a pathogen-free environment for at least two days before use.

Medium

RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), glutamine (5 mM), 100 U/ml penicillin, and 1 mg/ml streptomycin was used throughout the study. The medium is in the following text referred to as common medium.

Factors

Purified human recombinant (hr) IL-2 from *E. coli* (specific activity: 1.8×10^7 U/mg protein, from Cetus USA) was kindly provided by Dr. Kirchner, Medizinische Hochschule Hannover. Purified murine recombinant (mr) GM-CSF from yeast (specific activity: 5×10^7 U/mg protein) and human recombinant (hr) M-CSF from *E. coli* (specific activity: 1×10^7 U/mg protein) were generous gifts from Behring Werke AG, Marburg.

Tumor cell line

Yac-1, a Moloney virus-induced lymphoma of A/Sn mouse strain origin, was maintained as tissue culture in common medium and used as NK-sensitive tumor cell line for the measurement of NK/LAK-activities.

Antibodies

Monoclonal antibody (mAb) rat anti-mouse B220 (clone RA3-6B2) used for depleting $B220^+$ cells was purchased from Pharmingen. Fluorescein isothiocyanate (FITC)-labeled rat anti-mouse Ly-5 (B220 from clone RA3-6B2) obtained from Medac Hamburg was used for the fluorescent staining. mAb NK-1.1 from the tissue culture supernatant of hybridoma PK136 (obtained from American Type Culture Collection) was purified as described before [5] and used at 1:10 dilution. mAb F4/80 rat anti-mouse macrophage, also prepared from tissue culture supernatant, was used at 1:20 dilution. FITC-labeled mAbs hamster anti-mouse CD3,

rat anti-mouse Mac-1, rat anti-mouse Lyt-2 (CD8) and rat anti-mouse Thy-1.2 were obtained respectively from Medac and Becton Dickinson and used for the direct fluorescent staining. FITC-goat anti-mouse IgG, FITC-goat anti-rat IgG used for the indirect fluorescent staining were purchased from Dianova. mAb rat anti-mouse CD3 from SeroTec was used to deplete T cells. mAb CB.5.4 rat anti-murine perforin was kindly provided by Professor Tschopp, University de Lausanne, Switzerland.

BM preculture

Fresh bone marrow cells collected from the marrow of two femurs of each mouse were fractionated through a discontinuous Percoll-gradient to remove erythrocytes, mature granulocytes and small lymphocytes, which could not be activated by IL-2 to differentiate into NK cells as previously described [13]. Briefly, a 2-ml cell suspension (3×10^7 cells/ml in common medium) was carefully laid on a four-layer Percoll gradient (with Percoll concentrations of 38.0, 52.0, 55.0, and 60.6%) and spun at 550*g* for 30 min [6]. Cells from the 38.0/52.0% interface were collected, washed thrice, and then resuspended in common medium (supplemented with 5 μM 2-mercaptoethanol) at a density of $0.5\text{-}1 \times 10^6$ cells/ml. The cell suspension was plated in 9-cm tissue culture dishes (Greiner, Nürtingen) and incubated with 200 U/ml mr GM-CSF (as growth factor) and 100 U/ml hr IL-2 (as NK-activity stimulating factor) at 37°C in 5% CO_2 for 3 days. This short-period (3-day) bone marrow culture was named as BM preculture.

Isolation of nonadherent and nonphagocytic macrophage precursor (MP) cells from BM preculture

As previously described [6], nonadherent cells were harvested from BM preculture and fractionated on a six-layer discontinuous Percoll gradient (Percoll concentrations: 32.0, 40.8, 45.3, 50.0, 55.0, and 60.6%) at 550*g* for 30 min. Cells at the 40.8/45.3% interface was collected. This cell fraction consisted of 75-85% macrophage precursors (including a small number of semimature macrophages), 10-15% granulocytes, and 5-8% lymphocytes. Cells were washed, resuspended in common medium (1×10^6 cells/ml) and incubated with iron particles (Technicon Instruments, Tarrytown, NY; product no. T01-0507) in a plastic dish (Falcon 3003) for 30 min at 37°C to remove phagocytic and adherent granulocytes and semimature macrophages. Lymphocytes were depleted by incubating the cells at 4°C first with an antibody cocktail of rat anti-mouse B220 (for B cells) and rat anti-mouse CD3 (for T

cells) for 45 min, and then with the magnetic beads coated with sheep anti-rat IgG (Dynal A.S. Oslo) for 45 min. The beads were removed with Dynal magnet (Dynal MPC-1, Oslo). The remaining cells were purified nonadherent and nonphagocytic macrophage precursor cells.

Fluorescent staining and flow cytometric analysis

Cells for the staining were washed three times with staining buffer (PBS + 2% newborn calf serum + 0.01% NaN_3). For one sample, 2-5 × 10^5 cells were first incubated with 40 µl of human immunoglobulin solution (10 µg/ml) for 50 min on ice to block unspecific binding and then washed once. The cells were further incubated either in 40 µl of staining buffer alone or with 40 µl of antibody (FITC labeled or unlabeled) solution for 50 min on ice. The cells were washed twice and for those, which had been incubated with unlabeled antibody, an indirect fluorescent staining was done by incubating the cells with the relevant FITC antibody (1:40 diluted, 40 µl/sample) for 45 min on ice and then washed twice. The results of the staining were analyzed on a flow cytometer (FACScan, Becton Dickinson GmbH, Heidelberg).

Cytotoxicity assay

^{51}Cr-release assay using Yac-1 lymphoma as targets was performed to measure the cytotoxicity as previously described [6]. Briefly, effector cells were incubated in 96-well round-bottomed microtiter plates with ^{51}Cr-labeled Yac-1 cells (1 × 10^4 cells/well) in a final volume of 200 µl/well. The effector/target ratios were established as 5:1, 10:1 and 20:1. After 4 h incubation in 5% CO_2 atmosphere at 37°C, the plates were centrifuged (450*g*, 5 min) and 100 µl aliquots of the supernatants were collected and assessed for radioactivity. The spontaneous release was determined by incubating the target cells with medium alone. Total releasable counts were obtained from lysis with 1 N HCl. The percentage of specific ^{51}Cr-release was calculated as follows:

$$\% \text{ specific } {}^{51}\text{Cr-release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total releasable counts} - \text{spontaneous release}} \times 100$$

In the case of examining the cytotoxicity of the cell population, which was free from $B220^+$ cells, depletion of $B220^+$ cells using magnetic beads was performed right before the cytotoxicity assay. At 4°C, the cells were first incubated with rat anti-mouse B220 (Pharmingen) for 45 min and then with magnetic beads (coated with sheep anti rat IgG) for 45 min. The beads on which the $B220^+$ cells bound were finally removed with Dynal magnet.

Proliferation assay

[^{3}H]-Thymidine incorporation into replicating cells was measured to determine the proliferative ability of the cells. Cells were cultured in 96-well flat-bottomed microtiter plates (5×10^4 cells/well) and incubated at 37°C with relevant factors or medium alone for certain period. Then, [^{3}H]-Thymidine was added into the cultures (1μ Ci/well). After another 18-20 h incubation, the cells were harvested by a suction filtration apparatus (Skatron, Inc., sterling, VA). The [^{3}H] radioactivity bound to fiberglass filters was finally determined by β scintillation counter (Beckman LS1801).

Immunohistochemical double-staining

Peroxidase- and alkaline phosphatase-conjugated antibodies were used for the double staining. Cytospin preparations were air dried and fixed in 100% isopropanol for 10 min. After washing with TBS (100 mM Tris-HCl + 150 mM NaCl) supplemented with 0.1% Tween 20, the slides were soaked in the Methanol-H_2O_2-solution (250 ml Methanol + 5 ml of 30% H_2O_2 + 45 ml H_2O) for 10 min to inactive the endogenic peroxidase. The slides were then washed and preincubated with goat serum (1:10 diluted in washing buffer) for 40 min to block unspecific binding. After removal of goat serum, the first staining was carried out by incubating the slides with rat anti-mouse B220 for 50 min, followed by one time washing and further incubation with peroxidase conjugated goat anti-rat IgG (1:20 diluted in TBS) for 45 min. The slides were then thoroughly washed and incubated with the color-reaction-solution (10 ml of 70% 3,3-Diaminobenzidine in TBS + 4 μl of 30% H_2O_2) in dark for 5-15 min till the brown color developed. After rinsing with TBS plus 0.1% Tween 20, the slides were ready for the second staining. The second staining began with incubating the slides with antibody CB.5.4 rat anti-murine perforin (1:400 diluted in TBS) for 50 min. The slides were then washed thrice and incubated with alkaline phosphatase-conjugated goat $F(ab)_2$ anti-rat IgG (1:20 diluted in TBS) for 45 min. After three-time washing, the slides were incubated with Fast Blue-substrate solution (10 mg of Fast Blue RR in 9.8 ml of 0.1M tris buffer, pH

8.2, which contained 2 mg of naphthol AS-MX-phosphate and 0.01 ml of 1M levamisole) for 20-30 min. The reaction was stopped by rinsing the slides in distilled water. The final step was to immerse the slides in hematoxylin for 10 s for counterstaining, then washed in tap water, and air dried.

Statistical analysis

Differences measured in in vitro assays were analyzed by Student's t-test. All experiments were repeated at least three times.

RESULTS

MP cells from BM preculture

We have previously reported that the macrophage precursor (MP) cells isolated from a 3-day BM preculture supplemented with 200 U/ml GM-CSF (as growth factor) and 100 U/ml IL-2 (as NK activity-inducing factor) can differentiate into highly lytic cells if a further stimulation with high-dose (500-1000 U/ml) IL-2 is followed [6]. A close resemblance of these highly lytic cells to the reported NK/LAK cells was observed [6].

In the present work, to investigate if B220 marker could be expressed on the surface of these MP derived lytic cells, we first checked the antigenic profile of the MP cells that were freshly obtained from 3-day BM preculture and used as the initial population for cultivating NK/LAK effectors. Monoclonal antibodies: anti-mouse B220, NK-1.1, Mac-1, F4/80, anti-mouse CD3, CD8 and Thy-1.2 were chosen for the examination. Direct or indirect fluorescent staining was then performed.

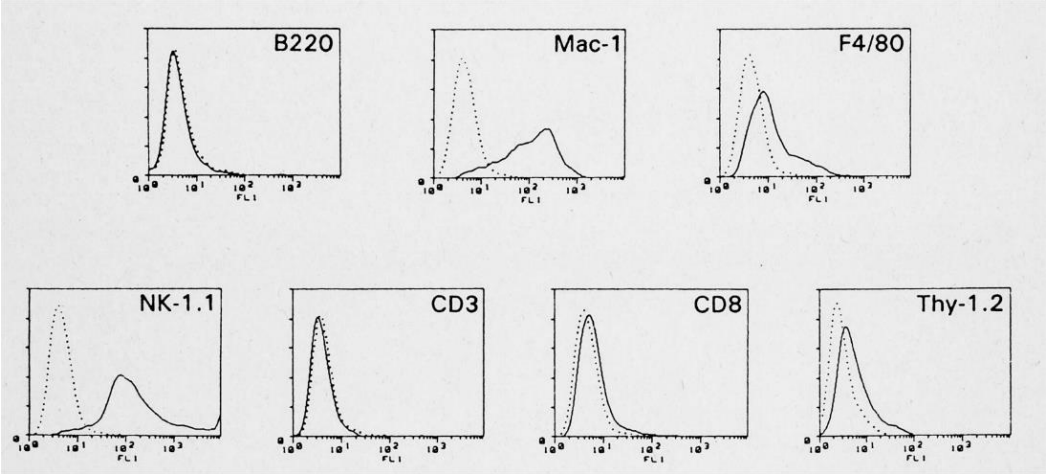

Fig. 1. Antigenic profile of the purified MP cells isolated from 3-day bone marrow preculture with GM-CSF + IL-2.

As shown in **Figure 1**, the macrophage precursor cells derived from 3-day BM preculture were completely negative for B220. The cells displayed an NK-like phenotype (NK-1.1^{+} CD3^{-} CD8^{-}) and were all positive for Mac-1 and weakly positive for F4/80, a known antigenic profile of macrophage precursors as we observed in the previous studies [14, 6]. By the way, this macrophage precursor population was found to be weekly positive for Thy-

1.2 marker. This finding corresponds to the report from Schrader et al. who observed that after a short period of tissue culture, Thy-1 antigen can be detected on a relatively large proportion of mouse bone marrow cells including myeloid cells together with hemopoietic stem and progenitor cells [15].

Induction of B220 expression on the MP cells in LAK-culture

$B220^-$ MP cells, obtained from BM preculture, were incubated with 200 U/ml M-CSF (as the factor to support MP cell survival and proliferation) + 500 U/ml IL-2 (as LAK activity-inducing factor). Under this culture condition, the MP cells can be stimulated as LAK effectors within 3 days [5, 6]. The culture condition is therefore referred to as LAK-culture.

To investigate whether B220 expression could be induced on the surface of MP cells during their development into LAK effectors, we chose 3 days and 5 days of LAK-cultures to harvest cells for the study, because we previously found that the LAK-activity in LAK-culture with MP cells is clearly detectable on day 3 and peaks by day 5-6 [5]. As control, a portion of $B220^-$ MP cells from BM preculture were parallelly cultivated with only M-CSF, a culture condition that is known to be not able to induce LAK-activity because of the lack of IL-2 [6].

Cells harvested from both cultures (M-CSF + IL-2 and M-CSF alone) were then stained with FITC-labeled anti-mouse B220 respectively. As expected, $B220^+$ cells were found in the culture with M-CSF + IL-2 (LAK-culture) , but not in the culture with only M-CSF (**Figure 2**). In LAK-culture, about 50% of all the cells were detected as $B220^+$ on day 3 (**Figure 2b**) and the percentage increased to 100% on day 5 (**Figure 2d)**. In contrast, culturing with only M-CSF did not result in any B220 expression throughout the whole 5-day culture period (**Figure 2c** and **2e**). These results indicate an important role of IL-2 in the induction of B220 marker on the MP cells.

Cells harvested on day 5 of both cultures (M-CSF + IL-2 and M-CSF alone) were also stained with F4/80 to check their expression of the surface marker of mature macrophages. As shown in **Figure 2g** and **2f**, the F4/80 expression was exclusively found on the cells from culture with M-CSF alone, while the $B220^+$ cells from LAK-culture were completely negative for F4/80. Interestingly, at these stages, we could neither induce any-B220 expression on the $F4/80^+$ cells by giving the cells with high-dose (500-1000 U/ml) IL-2, nor any F4/80 expression on the $B220^+$ cells by withdrawing IL-2 from LAK culture (data not shown).

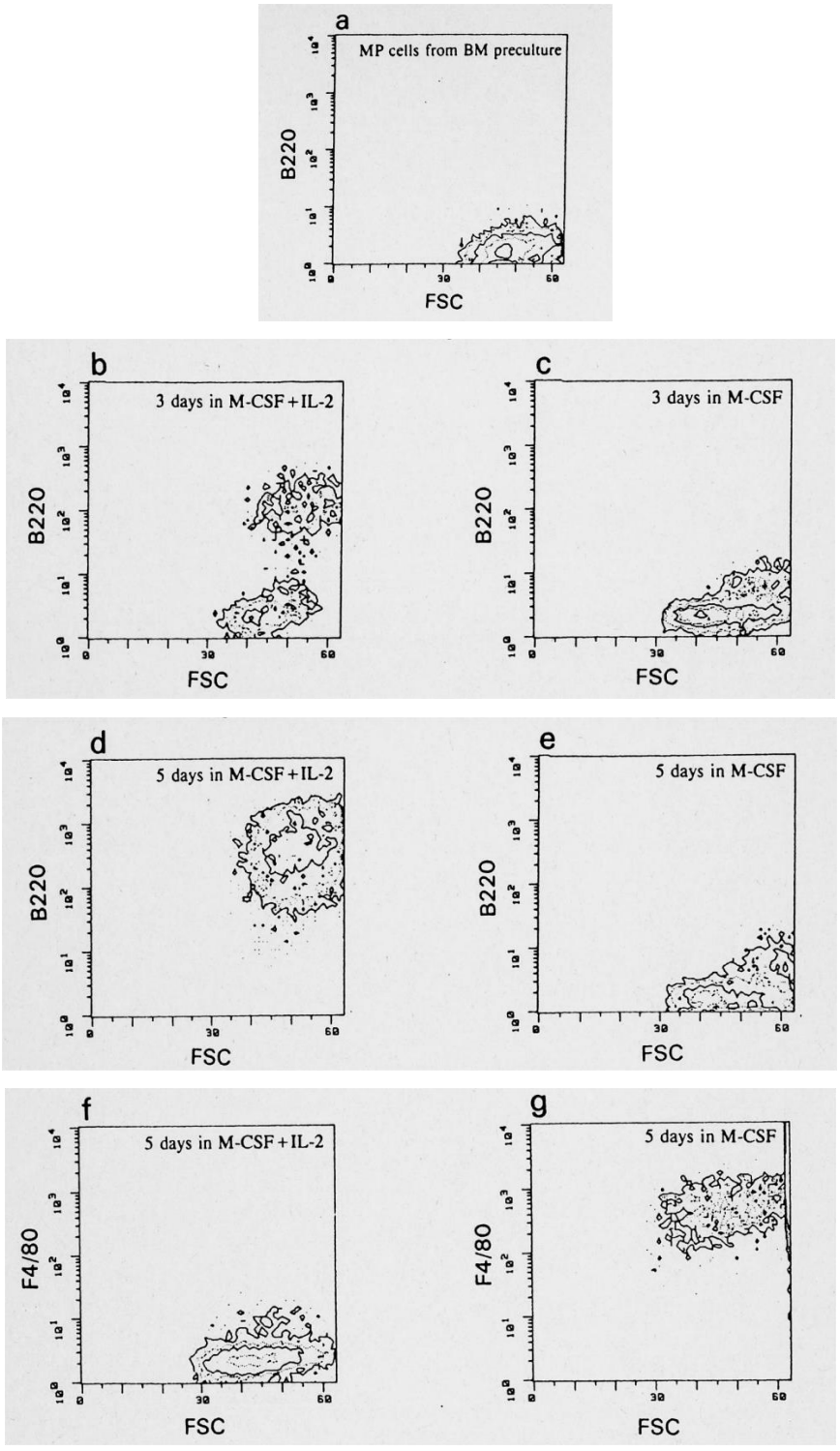

Fig. 2. Investigation of the expression of B220 and F4/80 markers on the cells harvested from different culture conditions. The purified $B220^{-}$ MP cells from bone marrow preculture (a) were divided into two portions and respectively cultivated in 200 U/ml M-CSF + 500 U/ml IL-2 and in 200 U/ml M-CSF alone. Fluorescent staining of the cells to check the expression of the markers was carried out after 3 days (b, c) and 5 days (d, e, f, g) of culture periods.

Growth factor for the precursors of B220$^+$ cells found in our LAK-culture

To exclude the possibility that the B220$^+$ cells we found in our LAK-culture might be expanded from contaminated lymphocytes, a proliferation assay to check the growth factor-dependence of the cells was carried out. The B220$^-$ MP cells that were used as the initial population for LAK-culture were seeded in 96-well microtiter plates (see Materials and Methods) and incubated with either medium alone or 500 U/ml IL-2 alone, or 500 U/ml M-CSF alone, or IL-2 + M-CSF (500 U/ml each). Factors IL-2 and M-CSF were chosen for the test because they were not only the factors we used for LAK-culture but also the well-known growth factors for lymphocytes (IL-2) and macrophages (M-CSF).

[^{3}H]-Thymidine incorporation assay was done after 2 and 3 days of the incubation to measure the proliferative activity of the cells. As shown in **Figure 3**, the most active cell proliferation was found in the cultures where M-CSF was added. IL-2 alone did not result in significant cell proliferation while M-CSF alone contributed almost so high cell proliferation as M-CSF + IL-2 did. The cells in our LAK-culture were therefore determined as M-CSF-dependent, so the B220$^+$ cells generated in our LAK-culture were derived from the M-CSF-dependent cells.

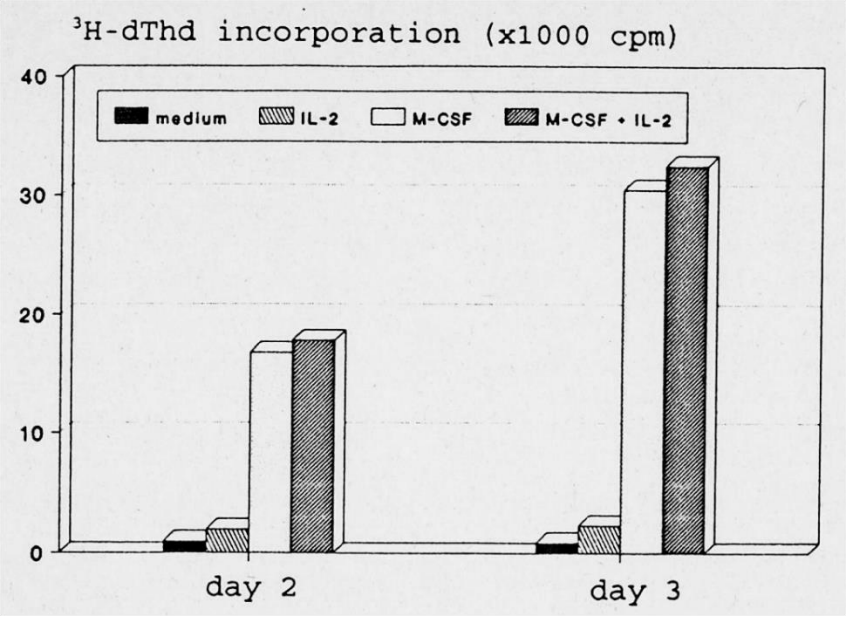

Fig. 3. Proliferation of the cells that could develop into B220$^+$ cells. MP cells from BM preculture were seeded into 96-well microtiter plates (5 × 10^4 cells /well in 200 µl medium) and incubated with either medium alone, or 500 U/ml IL- 2, or 500 U/ml M-CSF, or 500 U/ml IL-2 + 500 U/ml M-CSF. [^{3}H]-Thymidine incorporation was measured after 2 and 3 days of the incubation.

Correlation of B220 expression and cytolytic activities

Since the increase of the percentage of $B220^+$ cells in our LAK-culture seems to be parallel with the increase of LAK-activity observed previously [5], we supposed that these $B220^+$ cells found in our LAK-culture might, similarly to those found in the cultures with splenocytes [2,4], represent the actual effectors which exert the LAK-killing. To clarify this supposition, cytotoxicity of the cells harvested from a 3-day LAK-culture were assayed before and after depletion of $B220^+$ cells (see Materials and Methods).

We found that after removal of $B220^+$ cells, the cytolytic activity in the cell population drastically declined **(Figure 4).** This result shows that the cytotoxicity in our LAK culture was mostly contributed by the $B220^+$ cells. The population that had been depleted of $B220^+$ cells was further cultivated in the presence of IL-2 (500 U/ml). 3 days later, both $B220^+$ cells and cytolytic activity became again detectable in the cultures (**Table 1**). The cells could all developed into $B220^+$ cells within 4-5 days.

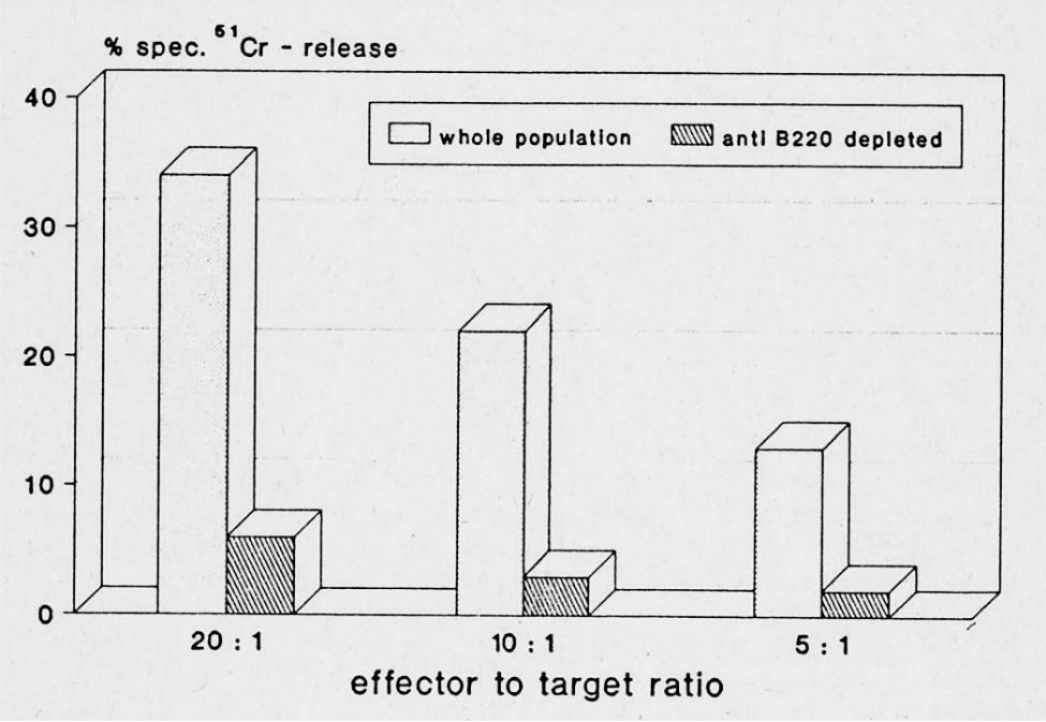

Fig. 4. Cytotoxicity of the cells from 3-day LAK-culture before and after depletion of $B220^+$ cells. ^{51}Cr-labeled Yac-1 cells were used as the targets. The incubation time of the effectors with targets lasted 4 hours.

Tab. 1. Re-Induction of B220$^+$ Cells and Cytotoxicity in the B220 Depleted Population [a]

Time of the Tests	Yac-1 Lysis (E:T = 20:1)	B220-Expression (% positive cells)
right after depletion	< 5 %	< 2
3 days after depletion	58 ±4%	45.3±2.7

[a] Cells from the population depleted of B220$^+$ cells were seeded into 96-well round bottomed microtiter plates (1×10^5 cells/well in 200 µl medium) and incubated with 200 U/ml M-CSF + 500 U/ml IL-2 for 3 days. Yac-1 lysis test was done in a 4-hour chromium-release assay and B220 expression was measured by fluorescent staining.

Antigenic profile of the B220$^+$ cells derived from MP cells

Since it was reported that under the LAK-culture conditions with murine splenocytes, several different cell-types, e.g. NK-1.1$^+$ CD8$^-$, NK-1.1$^-$ CD8$^+$ and NK-1.1$^-$ CD8$^-$ cells, can express B220 marker and develop into cytolytic effectors [4], we tested the antigenic profile of the B220$^+$ cells generated in our LAK-culture. As shown in **Figure 5**, only one cell population with the phenotype of B220$^+$ Mac-1$^-$ F4/80$^-$ NK-1.1$^+$ CD3$^-$ CD8$^-$ Thy-1.2$^+$ was found.

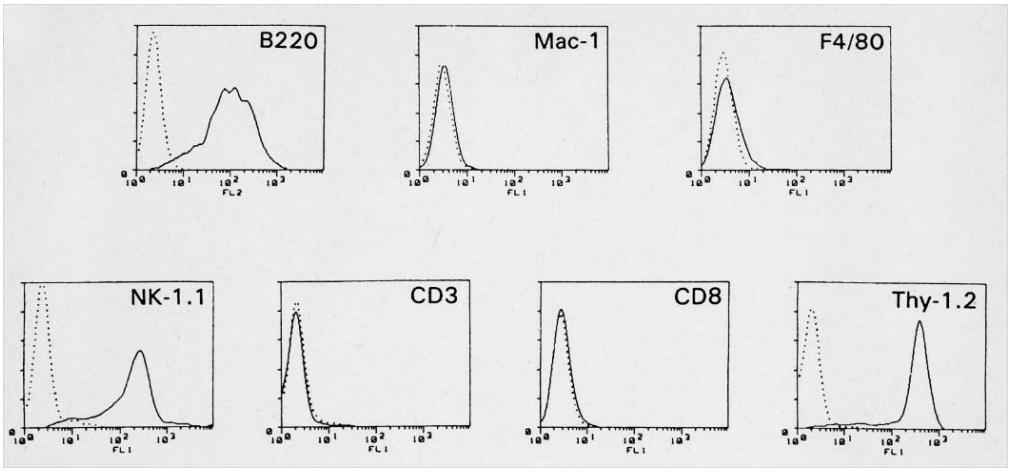

Fig. 5. Antigenic profile of the B220$^+$ cells harvested on day 5 of LAK-culture.

In comparison with the MP cells before LAK-culture **(Figure 1**), the $B220^+$ LAK effectors lost their myeloid marker Mac-1 and F4/80, while on the other hand their expression of Thy-1.2 marker became very strong (**Figure 5**). This type of antigenic profile resembles that of LAK subset derived from murine splenic NK cells [4, 16]. On the contrary, the expression of Mac-1, F4/80 and Thy-1.2 on the mature macrophages that were generated by cultivating the MP cells in M-CSF alone showed a completely different pattern (**Figure 6**). These mature macrophages kept NK-1.1 marker on their surface, expressed Mac-1 and F4/80 more strongly than their precursor stages, lost Thy-1.2 antigen (**Figure 6** v. **Figure 1**), and remained to be negative for B220, CD3 and CD8 (data not shown).

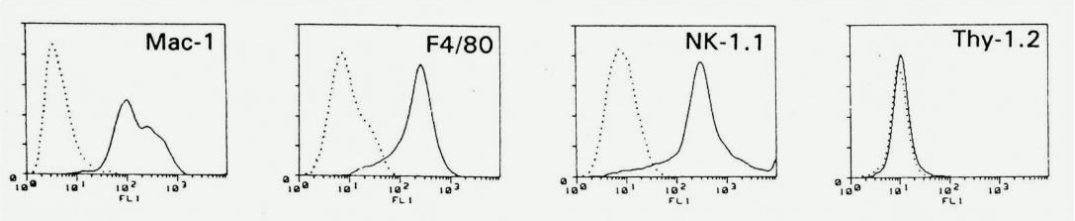

Fig. 6. Antigenic profile of the mature macrophages harvested on day 5 of M-CSF alone culture.

Investigation of the relationship between B220 expression and perforin production in MP cells

In our previous work, we noticed that the MP cells cultivated in M-CSF + IL-2 have significant difference to those cultivated in M-CSF alone through the test showing that the former ones produce perforin and the later not [6]. Since we had clarified it is perforin that endows the MP cells with NK/LAK-like cytotoxicity [6], we asked question, in the present work, if the expression of B220 marker could be related to the production of perforin protein in the macrophage precursor cells. To answer this question, immunohistochemical double-staining for B220 and perforin was performed (see Materials and Methods). Cells collected from day 4 of LAK-culture were examined.

As shown in **Figure 7**, the majority of the cells were found as positive for both antigens, but there existed some cells whose staining seems to be preferential only for one antibody, either for anti-B220 or for anti-perforin (see the cells indicated by arrows in **Figure 7**). Moreover, even in the double positive cells, the expression intensities of both antigens do not look like parallel. So, it is more likely that the expression of B220 marker and perforin production in

MP cells are separately regulated. Since under our culture conditions IL-2 is the single factor that results in the inductions of both proteins (B220 and perforin), it could be assumed that more than one induction pathways in the MP cells may be activated by IL-2. Further investigations need to be done to confirm this observation.

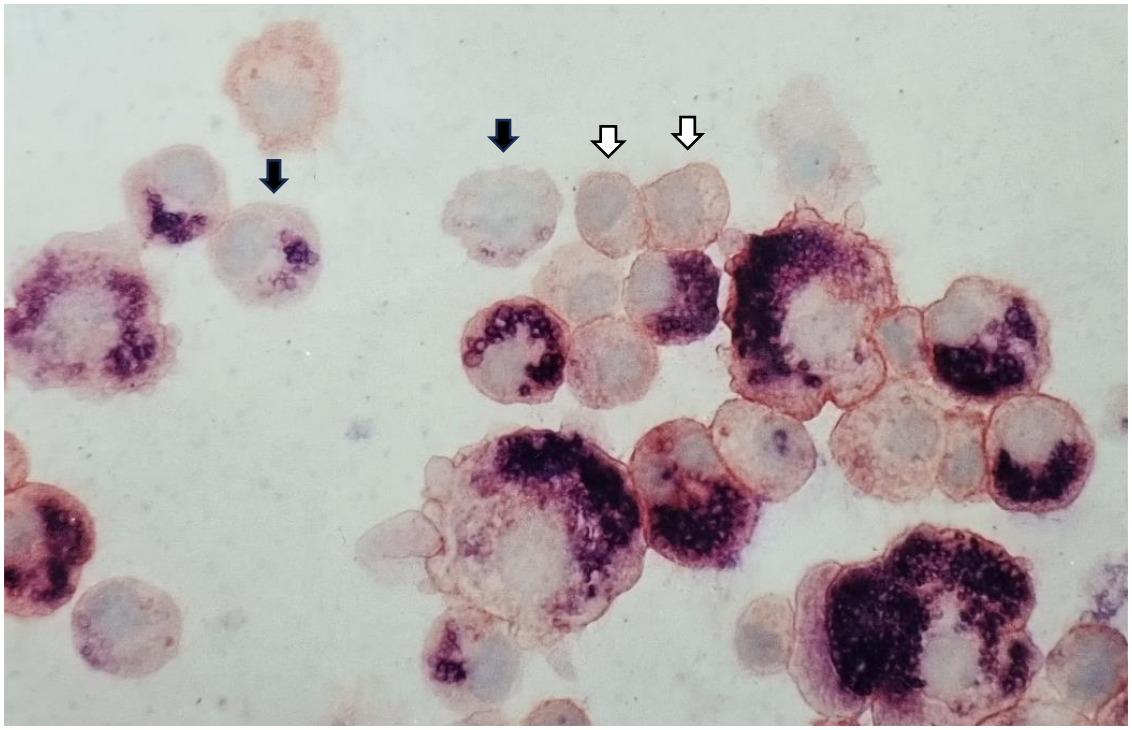

Fig. 7. Immunohistochemical double staining for B220 marker (stained as brown) and cytosol protein perforin (stained as blue). The cells were obtained from 4-day LAK-culture. The arrows (vacant for B220 and solid for perforin) point out the cells that appear to have been preferentially stained by only one antibody.

DISCUSSION

For some time, we have been focusing on studying the differentiation of young stages of macrophage lineage by virtue of bone marrow culture. We found that the bone marrow macrophage precursors appear to have two differentiation potentials, one to normal mature macrophages under the influence of M-CSF, and another, through activation with IL-2, to cytolytic cells which could not be distinguished from the reported NK/LAK cells based on the analyses of NK-1.1 marker, cytotoxicity [5, 13], and even the cytosol protein perforin [6]. In this work, we investigated if the IL-2 activated bone marrow macrophage precursors could also acquire B220 marker on their surface as the splenic NK/LAK cells do [2, 4]. The results in this work present an affirmative answer and show that in the respect of B220 expression, the IL-2 activated bone marrow macrophage precursors behave similarly to the splenic NK/LAK cells.

B220 marker is a restricted form of the CD45 (leukocyte-common Ag) family [17], which is known to be a protein tyrosine phosphatase [18]. The B220 epitope determined by mAb RA3-6B2 was shown to be B cell-specific [19, 20, 21]. This is still true in the cases where no any in vitro or in vivo activation has been done on the cells. However, upon activation with various stimuli, such as IL-2, IL-4 [2, 4], anti CD3 antibody [22, 23], concanavalin A [23], and staphylococcal enterotoxin B (SEB) [23], the expression of B220 marker (6B2 epitope) was widely found on T cells, including $CD3^+$ $CD4^-$ $CD8^-$ [22], $CD4^+$ [23], and $CD8^+$ [2, 4, 23] T cells, and also NK derived LAK cells [2, 4]. Therefore, the B220 has been recognized as a marker that is indicative of the activated stages of various cells, although its function is poorly understood. Based on the detailed studies on the relationship between cytolytic activities and B220 expression, Ballas & Rasmussen [2, 4] suggested that B220 acts as a marker of MHC-non-restricted cytotoxicity. However, a very recently published work [23] shows that the $CD4^+$ T cells also acquire B220 marker after activation. So, it seems not likely that B220 is only involved in the cytotoxicity.

In the present work, we observed that although the B220 is expressed on the most of lytic effectors derived from MP cells, the intensity of B220 expression is however not proportional to the amount of cytosol protein perforin (**Figure 7**), which has been shown in our previous work to be the central factor responsible for the cytolytic activities of the lL-2 activated MP cells [6]. We therefore conjecture that B220 marker may not be really needed for the perforin-

containing, MHC-non-restricted lytic cells to exert their cytotoxicity. More work needs to be done to clarify the role of this marker.

The results in this work, indicating that B220 marker also appears on the IL-2-activated macrophage precursors, provide us not only with new evidence showing the similarities of these cells to the cells of NK/LAK system, but also with noteworthy data implying that the expression of B220 marker may play a role in the variation of the antigenic profile of macrophage precursors during their differentiation. Ayoung stages, when the precursor cells are still potent to differentiate either into macrophages or into NK/LAK like cells, the myeloid marker Mac-1 is clearly detectable on the cell surface (**Figure 1**). However, after stimulation with high-dose IL-2, the cells start to express B220 marker and simultaneously the Mac-1 marker disappeared (**Figure 5** v. **Figure 1**). Likewise, the weak but distinguishable expression of F4/80 (marker for macrophage lineage) on the young stages (**Figure 1**) could no more be detected on the $B220^+$ cells (**Figure 5**). As a result, the cells changed into a population which lacks myeloid markers. This population kept to be positive for NK-1.1 and negative for CD3, CD8, and expressed Thy-1.2 marker much stronger than their young stages **(Figure 5** v. **Figure 1)**, demonstrating the same antigenic profile as that of LAK cells obtained by cultivating splenic NK cells in IL-2 [2, 4]. Therefore, we propose that this phenotypic variation of macrophage precursor cells observed in our work may represent the actual developing process of the NK/LAK cells that has long been remained as unclarified for their cell lineage.

It is worth noting that after the macrophage precursors had developed into $B220^+$ cells, they lost not only their myeloid markers but also the potential to differentiate into normal macrophages. The cells seem to have terminated their differentiation into the state of cytolytic cells and could no longer be converted into $F4/80^+$ mature macrophages (data not shown). Their proliferative activity in the presence of M-CSF began to decline dramatically (unpublished observation). We presume that high-dose IL-2 might be able to trigger a mechanism in the macrophage precursors that switches on the expression B220 marker and at the same time shuts off the expressions of Mac-1 and F4/80. Very likely this process is irreversible and it results in some basic changes in the properties of macrophage precursor cells. Studies based on molecular biological analyses need to be done to clarify this issue.

On the other hand, we observed that when the macrophage precursors had matured into normal macrophages in the culture with M-CSF alone (**Figure 6**), the cells seemed to have

lost the ability to express B220 marker, because regardless of 3-5 days of induction with even 1000 U/ml IL-2, the mature macrophages remained to be $B220^-$ (data not shown). This may imply that the surface marker switch from Mac-1, F4/80 to B220 we presumed above can only be induced at early differentiation stages of MP cells.

In conclusion, this report shows for the first time that B220 marker can be expressed on the cytolytic cells derived from bone marrow macrophage precursor cells. Based on the antigenic profile data obtained from this work, we suggest that the long-term controversy about the identity or lineage of NK/LAK cells resulted from inconsistent findings of the surface markers on NK/LAK cells [24, 25] might be due to the phenotypic variation of bone marrow macrophage precursors during their differentiation.

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