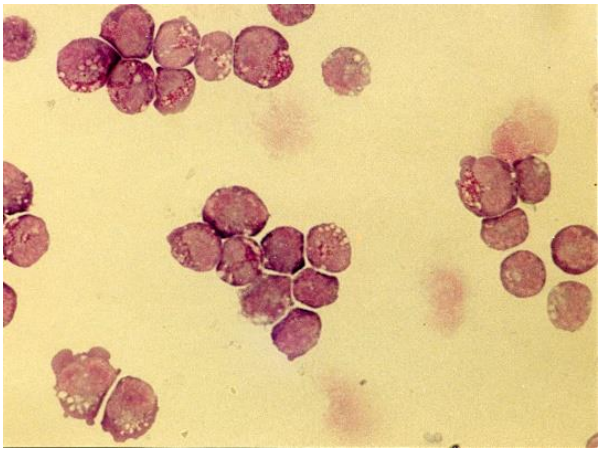
Differentiation of Bone Marrow Macrophage Precursors from Mouse under the Influence of Interleukin-2:

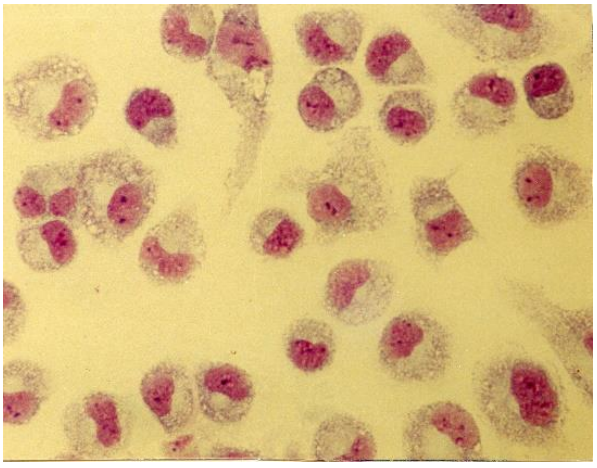
Are Interleukin-2 Activated Macrophage Precursors Natural Killer (NK-) Cells? [1]

(English version of my doctoral dissertation in German) [2]

Hao Li [3]



Macrophage precursors



Macrophages

Notes:

[1] Original title in German: Differenzierung von Makrophagen-Vorläufern des Knochenmarks bei der Maus unter dem Einfluss von Interleukin-2: Sind Interleukin-2 aktivierte Makrophagen-Vorläufer natürliche Killer (NK-) Zellen ?
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[2] This dissertation was completed in Germany in 1994, translated into English in 2023.

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Translation Preface

In 1994, I completed this doctoral dissertation in Germany and received my degree of Doctor of Natural Sciences at University Hannover (present name: Leibniz University Hannover). The major results of this dissertation had been published in international journals. In 1995, I came back to China and worked as a post-doctor at Beijing Medical University (present name: Peking University Health Science Center). Due to severe pollution problems in China at that time, in 1996, I left immunological research lab and became a voluntary educator for environment protection in China. Since then, I have been working for public environmental education till 2022.

In 2023, I translated my doctoral dissertation from German into Chinese and English for the following three reasons： (1) Nowadays, the functional diversity of macrophages has attracted attention of scientists, but there is still little known about the differentiation of macrophage precursors under the influence of cytokines. This dissertation shows that by activation of IL-2, within 3-6 days, murine bone marrow macrophage precursors can differentiate into NK cells, and later into LAK cells (if high dose IL-2 is given). The cell cytotoxicity is meditated by perforin, a lytic protein that can be induced in macrophage precursors by IL-2; (2) Cytokine storm is one of the main causes of severe symptom and death in COVID-19 epidemic. This dissertation shows that the IL-2 activated macrophage precursors can release 10 times more IL-6 and TNF-α (both are the major inflammatory factors found in cytokine storm) than the inactivated cells. In addition, high dose IL-2 can largely increase the cytotoxic granules in macrophage precursors, a few days later, the cells will fragment, releasing cytolytic granules (containing perforin) into surrounding. So, the IL-2 activated macrophage precursors may play a big role in cytokine storm and tissue damage; (3) Traditional Chinese medicine (TCM) gave a great help in prevention and treatment of COVID-19 infections in China. One of TCM's significant effects is to reduce the occurrence of cytokine storm. The cell culture conditions and cell differentiation behavior found in this dissertation may be useful in studying the mechanism of TCM or other drugs.

I hope the morphological, phenotypical and functional data of this dissertation can help the readers to expand their understanding of the differentiation process of macrophage precursors.

Dr. Li, Hao (李皓)

Beijing, China

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Differenzierung von Makrophagen-Vorläufern des Knochenmarks bei der Maus unter dem Einfluss von Interleukin-2:

Sind Interleukin-2 aktivierte Makrophagen-Vorläufer natürlich Killer (NK-) Zellen ?

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Von Fachbereich Biologie der Universität Hannover

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Last but not least, I thank my families from all my heart (my parent, my husband, my sister), although they are far from me, but have sent me steady love, encouragement, and support.

Parts of this work were already published:

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Li, H., E. Kniep, A. Emmendörffer and M.-L. Lohmann-Matthes: Differentiation of macrophage precursors to cells with LAK activity under the influence of CSF-1 and High Dose IL-2. Scand. J. Immunol., 33, 511-520, 1991.

Li, H., R. Schwinzer, M. Baccarini, and M.-L. Lohmann-Matthes: Cooperative effects of colony-stimulating factor 1 and recombinant interleukin 2 on proliferation and induction of cytotoxicity of macrophage precursors generated from mouse bone marrow cell cultures. J. Exp. Med., 169, 973-986, 1989.

ABSTRACT

It is well known that NK cells (natural killer cells) play an important role in the natural defense of immune system against neoplastic or virally infected cells, as well as certain microorganisms. Nevertheless, the cell lineage of NK cells has not yet been clarified. In the present work the bone-marrow precursor cells, which could differentiate into NK cells after activating with interleukin-2 (NK cytotoxicity stimulation), were studied under the bone marrow culture conditions. It was found out that these NK cell precursors from bone marrow were dependent on myeloid growth factors: GM-CSF (granulocyte-macrophage colony-stimulating factor) or CSF-1 (colony-stimulating factor 1, i.e. macrophage colony-stimulating factor), to proliferate. Incubating these NK cell precursors only with CSF-1 or PMA (phorbol 12-myristate 13-acetate), the factor which stimulates the cell differentiation, the cells developed into typical mature macrophages in a short time (24-72 hours). Thus, these bone marrow precursor cells, which were able to differentiate into NK cells, were identified as macrophage precursors.

Interleukin-2 is the crucial factor that influences the differentiation of bone marrow macrophage precursors into the direction to NK cells. Within 3-6 days of incubation with interleukin-2, these macrophage precursor cells developed first into NK cells and then into LAK cells (lymphokine-activated killer cells) which have been well known as the further activated form of NK cells. The cell types were determined by morphological, phenotypic, and functional investigations.

The experimental results presented in this work demonstrate that NK/LAK cells might derive from macrophage system.

Key words:* *murine bone marrow culture; NK cells; macrophage precursors: IL-2; CSF; LAK cells; perforin; cytokine release; differentiation of macrophage system*

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* "Key words" did not appear in the original German dissertation.

ABSTRAKT

(Abstract in German)

Es ist seit langen bekannt, dass NK-Zellen (natürliche Killer-Zellen) bei der natürlichen Abwehr des Immunsystems gegen neoplastische oder Virus-infizierte Zellen und manche Mikroorganismen eine wichtige Rolle spielen. Dennoch ist die Zell-zugehörigkeit der NK-Zellen immer noch nicht geklärt. In der vorliegenden Arbeit wurden mittels der Knochenmark-Kultur die Knochenmark-Vorläuferzellen untersucht, die sich durch Interleukin-2-Aktivierung (NK-Zytotoxizität Stimulation) zu NK-Zellen differenzierten. Es stellte sich heraus, dass diese NK-Zell-Vorläufer aus dem Knochenmark zur Proliferation von myeloischen Wachstumsfaktoren: GM-CSF (Granulozyten-Makrophagen Kolonie-stimulierende Faktor) oder CSF-1 (colony-stimulating factor 1 bzw. Makrophagen-Kolonie-stimulierende Faktor), abhängig waren. Wurden diese NK-Zell-Vorläufer allein mit CSF-1 oder PMA (Phorbol 12-Myristate 13-Acetate), welches die Differenzierung stimuliert, inkubiert, entwickelten sie sich in kurzer Zeit (24-72 Stunden) zu typischen reifen Makrophagen. Deshalb wurden diese Knochenmark-Vorläuferzellen, die fähig waren, zu NK-Zellen zu differenzieren, als Makrophagen-Vorläufer identifiziert.

Interleukin-2 ist der entscheidende Faktor, der die Differenzierung der Makrophagen-Vorläufer des Knochenmarks in die Richtung zu NK-Zellen beeinflusst. Bei der Inkubation mit Interleukin-2 konnten sich diese Makrophagen-Vorläuferzellen innerhalb von 3-6 Tagen zunächst zu typischen NK-Zellen, danach zu deren weiter aktivierten Form LAK-Zellen (Lymphokin-aktivierte-Killer-Zellen) entwickeln. Die Zelltypen wurden durch morphologische, phänotypische, und funktionelle Untersuchungen bestimmt.

Die Ergebnisse aus dieser Arbeit deuten an, dass NK-/LAK-Zellen wahrscheinlich aus dem Makrophagen-system abstammen.

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LIST OF ABBREVIATIONS

Ab	antibody
AP	alkaline phosphatase
bidist.	Bidistilled (not shown in the original German dissertation)
BM	bone marrow
BSA	Bovines serum albumin
°C	degree centigrade
cDNA	complementary desoxyribonucleic acid
CFU	colony forming units
Ci	Curie
CM	complete medium
Con A	concanavalin A
cpm	counts per minute
^{51}Cr	Chrom-51 (sodium chromate)
CSF	colony-stimulating factor
DAB	3,3′-diaminobenzidine tetrahydrochloride
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
dist.	distilled
DMF	N, N-dimethylformamide
EDTA	ethylenediamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCS	fetal calf serum

FITC	fluorescein isothiocyanate
g	gravity
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour
Hepes	N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]
hr	human recombinant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-2 R	interleukin-2 receptor
Ion.	ionomycin
LAK	lymphokine-activated killer
L cells	L929 fibroblast cells
LGL	large granular lymphocytes
LU	lytic units
M	molar
mAb	monoclonal antibody
M-CSF	macrophage colony-stimulating factor
2-ME	2-mercaptoethanol
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mr	mouse recombinant (not shown in the original German dissertation)

mRNA	messenger-ribonucleic acid
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromides
MP	macrophage precursor
Mϕ	macrophage
NBT	nitro blue tetrazolium salt
NK	natural killer
PBS	phosphate-buffered saline
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear neutrophile leukocytes
POD	peroxidase
rpm	rotations per minute
RT	room temperature
SDS	sodium dodecyl sulfate
sec.	second
spec.	specific
SR	spontaneous release
TBS	Tris-buffered saline
TCR	T cell receptor
TE	Tris-EDTA
TMS	Tris-magnesium saline
TNF	tumor necrosis factor
Tris	Tris-hydroxymethyl-aminomethane
U	unit

1. INTRODUCTION

1.1. NK Cells and Macrophage precursors

20 years ago (1972-1973, translation notes), several groups of scientists accidentally discovered the natural killer cells during their study of cell mediated cytotoxicity [Rosenberg et al. 1972; Takasugi et al. 1973]. The researchers wished to find a specific cytotoxic activity of cells from tumor bearing patients against autologous tumor cells or against allogeneic tumors of similar or the same histological type, and they found a cytotoxicity of " lymphocytes" from normal individuals. This kind of cytotoxic activity is different from the activity of cytotoxic T lymphocytes by the following characteristics: (1) It does not show any MHC restriction [Kiessling et al. 1975]; (2) It does not appear to be dependent on the antigenic sensitization and does not show a specific "secondary memory" response [Herberman et al. 1975]. These cytotoxic cells can spontaneously destroy the so-called NK target cells which are sensitive to this form of cytotoxicity, such as syngeneic, allogeneic, and xenogeneic tumor cells (leukemia cells, carcinoma cells and other types [Herberman & Holden 1978]), virus infected cells (e.g. herpes and influenza virus [Biron & Welsh 1982]), and even some microorganisms [Nencioni et al. 1983; Morgan et al. 1984]. Therefore, this spontaneous cytotoxic activity was named as "natural killing" activity and the effector cells responsible for it were called NK cells. Immediately after their discovery, functional experiments in the *in vivo* model showed that NK cells play an important role in natural resistance against tumors and infectious diseases [Baldwin et al. 1976; Herberman & Holden 1978; and Hanna et al. 1980, 1981].

A great deal of researches on NK cells, mainly on their identity, origin and belonging, were made in the first decade after their discovery. Although at that time it was not possible to isolate pure NK cells, the following properties were consistently determined in the cells that exerted NK activity: (1) NK activity is mediated by cells with LGL

(large granular lymphocytes) morphology; (2) NK effector cells are nonadherent and nonphagocytic cells that are most frequently found in peripheral blood and spleen; (3) The NK effector cells lack typical properties and surface markers of classical macrophages, granulocytes, B cells or T cells [Overview by Ortaldo & Herberman 1984; Tutt et al. 1986]. Thanks to the production of specific monoclonal antibodies anti-NK cells and the application of recombinant factors in cell culture, since 1986 the methods for isolation, culture and analysis of NK cells were essentially improved and obtaining NK cells with high purity was made possible [Hackett et al. 1986]. In 1988, Fitzgerald-Bocarsly et al. published the first precise definition of NK cells [Fitzgerald-Bocarsly et al. 1988]. It was described that NK cells are $CD3^-$, TCR^- large granular lymphocytes and that on their surface they express specific markers (by human CD16 as well as NKH-1 and by mouse NK-1.1 or NK-2.1). NK cells do not need the expression of MHC class I or II molecules for their cytolytic reaction. This is a significant difference between NK cells and T cells, but it has not sufficiently clarified the belonging of NK cells.

Through a transplantation experiment by Haller et al. in 1977, it was determined that the precursors of NK cells come from bone marrow [Haller et al. 1977]. 10 years later, it could be shown by *in vitro* investigation that IL-2 is the essential factor that stimulates the development of these bone marrow precursors from NK-inactive form to NK-active effector cells [Koo et al. 1986; Pollack et al. 1987; and Lotzova et al. 1987]. In mouse system, Hackett et al. found that the bone marrow precursors for NK cells express NK-1.1 marker [Hackett et al. 1986]. Whether these precursors represent an independent cell type or whether they belong to an already known cell type is still unclear till today (in 1994, translation notes).

In 1979, the first publication was published that reported on the similarities between

NK cells and promonocytes, also called macrophage precursors [Lohmann-Matthes et al. 1979]. These promonocytes were isolated from a 5-day mouse bone marrow culture in L cell-conditioned medium and were nonadherent and nonphagocytic cells. They were cytolytic to the NK target cells Yac-1 and looked like medium-sized lymphocytes. They were identified as promonocytes because they were able to differentiate into macrophages in CSF-1 containing L cell-conditioned medium. The authors hypothesized that these macrophage precursor cells are related to the described NK cells, or that in both cases it could be dealt with the same cells. This novel view was later supported by experiments with macrophage precursors isolated from spleen and liver [Baccarini et al. 1985, 1986a, 1986b; Decker et al. 1986]. However, the hypothesis that NK cells probably come from the macrophage system was ignored for a long time, because other authors showed that no NK activity was found in bone marrow culture only with purified macrophage growth factor CSF-1 [Koo & Manyak 1986].

From 1988 to 1993, the number of publications from the research on the belonging of NK cells is low. Searching for NK precursors in bone marrow was mostly performed with "LTBMC" ("long-term bone marrow culture") culture [Van-den-Brink et al. 1990; Sitnicka & Hansson 1992; Vecchini et al. 1993]. In these long-term bone marrow cultures, the whole bone marrow cells are first incubated for 4 weeks without exogenously added factors, then cultured with IL-2 for another 3-6 days. The NK activity appears in the culture approximately after 3-day incubation with IL-2. About the precursor cells, which are responsible for the generation of NK activity, all we known is that they are different from T cell precursors [Lotzova et al. 1993].

1.2. IL-2 and IL-2 Receptors

As mentioned above, IL-2 is the essential factor that stimulates the NK activity in bone marrow cells. This function of IL-2 was found through researches with bone marrow

cells from mouse, human, and rats [Koo et al. 1986; Lotzova & Savary 1987; and Van-den-Brink 1990]. This is considered to be one of the most important effects of IL-2 on the immune system.

Originally, IL-2 was discovered in the supernatant of human peripheral blood lymphocytes that had been incubated with T cell specific mitogens [Morgan & Ruscetti 1976]. Functional and phenotypical data suggested that the factor promotes the growth of T lymphocytes [Ruscetti et al. 1977]. It turned out later that the activities of following factors are all due to the same molecule: (1) T cell growth factor (TCGF); (2) Thymocytes stimulating factor (TSF); (3) Thymocytes mitogenesis factor (TMF); (4) T cells replacing factor (TRF); and (5) Killer helper factor (KHF). This molecule was named Interleukin-2 (IL-2) after the second international "Lymphokine Workshop" in 1979 [Aarden et al. 1979].

IL-2 is a protein composed of 133 amino acids (molecular mass 15,420) and shows no sequence relationship to other factors [Taniguchi et al. 1983; Kashima et al. 1985]. It exerts its effects through specific, saturable receptors, which are found on the surfaces of T cells, B cells [Overview by Smith 1989], NK/LAK cells [Siegel et al. 1987], monocytes [Wahl et al. 1987; Holter et al. 1987; Espinoza-Delgado et al. 1990] and neutrophiles [Djeu et al. 1993]. Distinctions are made among three different types of receptors, which can be expressed independently from one another. The high affinity IL-2 receptor ($K_d \approx 10^{-11}$ M) is a complex of two protein chains of IL-2R α (p55, called Tac antigen in human system) and IL-2R β (referred to as p75 or p70) [Sharon et al. 1986; Teshigawara et al. 1987]. The IL-2 receptor of intermediate affinity ($K_d \approx 10^{-9}$ M) consists only of the β-chain, while the α-chain represents a receptor of low affinity ($K_d \approx 10^{-8}$ M) [Tsudo et al, 1986; Teshigawara et al. 1987; and Robb et al. 1987]. In activated T cells, the stimulation by IL-2 occurs after binding to the high affinity receptor [Tsudo et al. 1987]. In NK cells, however, a signal transduction by binding

alone to the β subunit was observed, in which NK cells reacted to IL-2 in the absence of Tac antigen (α subunit) and became cytolytic [Siegel et al. 1987; Tsudo et al. 1987]. The mechanism, how the signal transduction occurs in the activation by IL-2, is not clear. It is generally supposed that the phosphorylation state of the receptor plays a key role [Sharon et al. 1989].

Baccarini et al. reported in 1989 that the p75 IL-2 receptor is also present on murine macrophage precursors from the bone marrow [Baccarini et al. 1989]. Similar to NK cells and to the non-activated monocytes and neutrophiles, only the IL-2R β subunit (p75) is found on the surface of these murine promonocytes.

For some time, it has been known that in addition to its long-known effect on the growth of T cells, IL-2 has several other effects on various cells of immune system, for example: (1) It activates the tumor killing cytotoxicity and the growth of NK/LAK cells [Herbermann et al. 1987]; (2) It increases the proliferation of B cells and their production of immunoglobulin [Ceuppens et al. 1985]; (3) It stimulates monocytes (in human) to increase their cytotoxicity [Malkovsky et al. 1987] and to release IL-6 [Musso et al. 1992]; (4) It modulates the release of histamine from activated basophiles [White et al. 1992]. Therefore, IL-2 is no longer considered as a specific factor that only acts on lymphocytes.

1.3. LAK Cells and Their Relationship to NK Cells

In 1982, Grimm et al. observed a previously undescribed cytotoxic activity, namely LAK (lymphokine-activated killer) activity, in *in vitro* culture of human peripheral blood lymphocytes with IL-2-containing supernatants. LAK activity differs from NK activity in that it lyses both NK-sensitive and NK-resistant tumor cells [Grimm et al.

1982]. This finding immediately attracted a lot of attention because it raised the hopes for a new tumor therapy. Some research groups began right away to experiment with the LAK cells cultivated *in vitro* in the animal models [Rosenstein et al. 1984; Mule et al. 1984; and Lafreniere & Rosenberg 1985]. IL-2 is, once again, been confirmed as the crucial factor that induces the LAK activity [Rosenberg et al. 1984]. Yang et al. described later in more detail that an effective *in vitro* culture requires high concentration of IL-2 (1000 U/ml) for the generation of LAK activity and that the culture usually lasts from 72 to 96 hours [Yang et al. 1986].

In the earliest years after the discovery of LAK phenomenon, the opinions about the precursor cells for LAK activity were incompatible [Grimm et al. 1982, 1983; Karre et al. 1983; and Rosenstein et al. 1984]. The reason is that the cultures at that time never reached a high purity. It was only after the application of the positive sorting technique, the research results from mouse [Salup et al. 1987], human [Morris et al. 1989], and rats [Maghazachi et al. 1988; Vujanovic et al 1989] agreed first that the cells, which developed LAK activity under the influence of IL-2, are NK cells. With other words: the most LAK effectors are the NK cells activated by IL-2. The main characteristics of these LAK effectors are: (1) They do not express T cell markers, such as CD3 and CD8; (2) They mediate MHC-independent cytotoxicity against both NK-sensitive and NK-resistant tumor cells [Fitzgerald-Bocarsly et al. 1988].

In the investigations with bone marrow cultures, consistent results prove that NK activity and LAK activity are generated from the same, non-cytotoxic bone marrow precursor cells by IL-2 stimulation, although the two activities were detected after different times. LAK activity appears in the culture a bit later (~3 days) than NK activity [Sarneva et al. 1989; Keever et al. 1990; Sitnicka & Hansson 1992]. These results have confirmed the relationship between NK cells and LAK cells as two differentiation states at the bone marrow precursor level.

1.4. Mechanism of NK/LAK Cytotoxicity and the Membrane Hole-Forming Perforin

Although NK/LAK cells are clearly different from cytotoxic T cells, because they do not carry typical surface markers for cytotoxic T cells, such as CD3 and CD8, and they have cytolytic activity independent of the expression of MHC molecule, on the other hand, they have something in common with cytotoxic T cells, namely in the cytoplasm of both types of cells the lytic protein, the so-called perforin, is found, which is normally not observed in other cells, such as monocytes and macrophages, granulocytes, B cells and T helper cells [Masson & Tschopp 1985; Podack et al. 1985 a and b; Young et al. 1986; Liu et al. 1986; and Lowrey et al. 1988].

The first reports on perforin appeared in 1984, when some research groups became interested in the cytoplasmatic granules from cytotoxic T cells and LGL cells [Millard et al. 1984; Henkart et al, 1984; Podack et al. 1984; and Blumenthal et al. 1984]. The researchers found that these granules, isolated from cytotoxic cells, can make membrane permeable on their own. Therefore, the researchers have named the lytic component in the granules differently as "cytolin", "pore forming protein", or "C9-related protein" according to its function. One year later, the name "perforin" was agreed upon [Podack et al. 1985a]. In numerous biochemical studies, it has been found that perforin is a protein that consists of 534 amino acids and exists only in cytotoxic granules. Together with other enzymes in granules, perforin plays an essential role in the killing to target cells by cytotoxic effector cells [Tschopp & Nabholz 1990; Young et al. 1990].

Unlike lymphotoxin and TNF, whose cytotoxic function represents only one of their pleiotropic activities, perforin is a protein with a single function: to lyse the membrane of the target cells and to lead them to cell death [Podack et al. 1991]. The lysis, which

is associated with perforin, is conditioned on the presence of calcium. In the presence of calcium (~1 mM), perforin can successfully polymerize on the membrane of the target cells, and then lyse the target cells. Each finished perforin polymer, also referred to as poly-perforin, consists of 12-18 monomers and is located in the target membrane as a closed cylinder of 16 nm in length and 5-20 nm in diameter [Tschopp & Nabholz 1990].

All CD8 positive CTL (cytotoxic T lymphocyte, translation notes) cell lines and all NK cell populations (including LAK cells) contain perforin [Podack et al. 1991]. In the low-perforin expressing, freshly isolated $CD8^+$ T cells from human peripheral blood, the amount of perforin mRNA was rapidly increased after IL-2 activation [Lichtenheld et al. 1988; Smyth et al. 1990a]. IL-2 alone is enough to induce high level of perforin mRNA. The activation is done via the p75 IL-2R. This process is independent of the existence of T cell receptor [Liu et al. 1989; Smyth et al. 1990a]. Interesting but still incomprehensible is that the IL-2 induced perforin increase can be enhanced by IL-6, although IL-6 alone is completely ineffective [Smyth et al. 1990b]. Perforin does not appear to be inducible in B cells and monocytes [Podack et al. 1991].

1.5. Regulatory Function of NK Cells

NK cells are important immunoregulatory cells, because they are able to release a lot of cytokines. Although in earlier studies of NK cells (1981-1983), the researchers had already observed that several types of lymphokines, such as alpha and gamma IFN, IL-1, IL-2, lymphotoxin (LT), colony-stimulating factors, and B cell growth factor, were found in supernatants of NK cell culture [Overview by Ortaldo & Herberman 1984], they could not rule out the possibility that the cells contaminating the NK cell culture, for example T lymphocytes, were responsible for the release of cytokines. Since 1986,

when the obtaining of highly purified NK cell populations and specific stimulation of NK cells (for example: with specific ligand) became possible, the ability of NK cells to produce cytokines had been confirmed in various laboratories [Overview by Trinchieri 1989].

Both freshly isolated and cultured NK cells can synthesize, either spontaneously or after stimulation, a number of cytokines, which were compiled in the **Table** "Cytokine Production of NK Cells" (see **Tab. A** on page 10) with data from different laboratories. The investigations for the ability of NK cells to produce cytokines were performed either by Northern blot analysis or polymerase chain reaction (PCR) at messenger RNA level, or by activity assay or immunoprecipitation at protein level. For activation of NK cells, the following stimulators were often used: NK target cells, CD16 FcR ligand (for example: Sepharose connected anti-CD16 antibodies or immune complexes, such as erythrocytes or target cells covered with IgG antibody), IL-2, IFN-γ, and phorbol ester/calcium ionophore. A combination of two stimulators was often used, because it works clearly more effective than a single factor. In human system, for example, the combination of CD16 ligand with IL-2 showed very effective, because under this stimulation condition, within 2 hours, active synthesis of messenger RNA for TNF, IFN-γ, GM-CSF and CSF-1 could be detected in NK cells. In contrast, the CD16 ligand alone had almost no effect at all, and IL-2 alone caused only a weak induction of GM-CSF mRNA [Cuturi et al. 1989]. In the experiments with NK target cells alone or plus IFN-γ as an activator, significant higher TNF release was observed in the combination [Peter et al. 1986]. The mechanism of synergistic action of two stimulators is not yet clear.

Tab. A. ***Cytokine Production of NK Cells***

(Data from the Literature)

Stimulator	**Synthesized Cytokine**	**mRNA$^+$**	**Protein$^+$**
None	IL-1-β	1	
	IL-6	1	
NK target cells	IL-1		2
	TNF (low)		3
NK target cells + IFN-γ	TNF (high)		3
IL-2	GM-CSF (low)	5	5
	IL-6	1	
IL-2 + CD16 FcR Ligand	CSF-1	4,5,6	6
	G-CSF	6	6
	GM-CSF	4,5,6	5,6
	IFN-γ	4,5,6	5,6
	TNF	4,5,6	5,6
IL-2 + Immunocomplex	IFN-γ		5
	TNF		5
Phorbol Dibutyrate und/or Calcium Ionophore A23187	GM-CSF	4,5	5
	IL-3	4,5	
	TNF	4,5	

1. Vitolo et al. 1993
2. Herman et al. 1986
3. Peters et al. 1986
4. Anegon et al. 1988
5. Cuturi et al. 1989
6. Saito et al. 1993

1.6. Aim of This Work

1. It should be tried to establish the conditions for primary bone marrow cultures, under which the precursors for NK cells present in bone marrow can both proliferate and develop into NK-active effectors. Thereby the growth factor for these precursor cells should be defined.

2. An isolation method should be established, with which the cultivated NK-active cells from bone marrow culture can be purified to a high degree. The isolated NK cells should be investigated and characterized at morphological, phenotypical, functional, and differentiating levels.

3. If the NK-active cells obtained from bone marrow culture are identical to the macrophage precursors, it should be further clarified whether these cells are differentiable to LAK effectors, and whether perforin is found in their cytoplasm as in the described NK/LAK cells.

4. Since IL-2 has been recognized as a very important stimulating factor for NK/LAK activity and IL-2R is present on murine macrophage precursors from the bone marrow, in this study, the cytotoxicity-stimulating effect of IL-2 on macrophage precursors should be studied at functional and gene levels.

5. The question, what similarities and differences there are between macrophage precursors and NK cells, should be answered by this work in the areas of morphology, phenotype (surface antigen profile), cytotoxic function, cytotoxic mechanism, and cytokine production spectrum.

2. MATERIALS AND METHODS

Materials

2.1. Animals

Six to eight-week-old C57BL/6 mice were obtained from the Breed of Charles River, Sulzfeld, or from the Central Laboratory Animal Facility, Hannover.

2.2. Chemicals

For Cell Isolation and Cell Culture

Agar (bacteriological degree)	Co. OXOID, Basingstoke	L11
Concanavalin A	Co. Pharmacia, Freiburg	17-0450-01
FCS (fetal calf serum)	Co. Sigma, Deisenhofen	F-7524
Ficoll-Paque (sterile)	Co. Pharmacia	17-0840-02
α-D-glucose	Co. Serva, Heidelberg	22720
Hepes (N-2-Hydroxyethyl-piperazine-N'-2-ethane sulfonic acid. pKa. 20°C = 7.55)	Co. Biomol, Hamburg	05288
Iscove's Medium	Co. Gibco-BRL, Karlsruhe	074-02200A
2-ME (2-mercaptoethanol)	Co. Sigma	M-7154
Percoll (sterile)	Co. Pharmacia	17-0891-01
RPMI 1640	Co. Gibco-BRL	074-01800N
Sodium pyruvate	Co. Biochrom	L0473

For Cell Fixation, Staining and Embedding

Aquatex	Co. Merck, Darmstadt	Art.No.8562
Bovin serum albumin	Co. Sigma	A-3350

DMF (N, N-Dimethylformamide)	Co. Sigma	D-4254
Ethylene glycol monomethyl ether	Co. Merck	Art.No.859
Fast Garnet GBC	Co. Sigma	F-6504
Fast Blue BB Salt	Co. Sigma	F-3378
Fast Red TR	Co. Sigma	F-2768
Gelatine (for microbiology)	Co. Merck	Art.No.4070
Giemsa-Solution	Co. Sigma	GS-500
Glycerin	Co. Serva	23175
H_2O_2	Co. Merck-Schuchardt	822287
Hematoxylin	Co. Sigma	H-3136
Human Ig	Co. Sigma	I-4506
Isopropanol	Co. Roth, Karlsruhe	Art.6752
Levamisole	Co. Sigma	L-9756
Mayer's Hemalaun-Solution	Co. Merck	Art.No.9249
May-Greenwald-Solution	Co. Sigma	MG-500
NaN_3	Co. Riedel-deHaen	1687
Naphthol AS-D Chloroacetate	Co. Sigma	N-0758
Naphthol AS-MX Phosphate	Co. Sigma	N-4875
α-Naphthyl butyrate	Co. Merck	Art.No.502

For Different Tests

Actinomycin D	Co. Sigma	A-4262
Avidin-POD-conjugate	Co. DAKO, Hamburg	P 347
DAB (3,3'-Diamino-benzidine-tetrahydrochloride)	Co. Sigma	D-5637
Ionomycin	Co. Sigma	I-0634
MTT (3-[4,5-Dimethylthiazol-2-yl] -2,5, -diphenyltetrazolium bromide	Co. Sigma	M-2128

PMA (Phorbol 12-myristate 13-acetate)	Co. Sigma	P-8139
Rotiszint 2200 (Scintillation fluid)	Co. Roth	Art. 9389
Tween 20 (Polyoxyethylene-sorbitan-monolaurate)	Co. Merck	822184

For Molecular Biological Work

Agarose (electrophoresis degree)	Co. Gibco-BRL	55100B
DEPC (diethyl pyrocarbonate)	Co. Sigma	D-5758
Dextran sulfate	Co. Pharmacia	17-0340-01
DIG-DNA Labeling Kit	Co. Boehringer-Mannheim	1175033
DIG-Nucleic Acid Detection Kit	Co. Boehringer-Mannheim	1175041
EDTA	Co. Sigma	E-4378
Ethidium bromide	Co. Serva	21238
Formamide	Co. Sigma	F-7503
Guanidinium isothiocyanate	Co. Stratagene, Heidelberg	300116
Megaprime DNA Labeling Kit	Co. Amersham	RPN.1607
Polyvinylpyrrolidone	Co. Sigma	P-5288
Salmon Sperm DNA	Co. Stratagene	201190
Sarcosyl	Co. Sigma	L-5125
Tris (research degree)	Co. Serva	37190
Triton-X-100	Co. Serva	37240
Yeast tRNA	Co. Sigma	R-8759

2.3. Cytokines

IL-1: Recombinant murine interleukin-1α from *E.coli* from Oncogene Science (specific activity of 1×10^7 U/mg protein) was obtained through company Dianova and used as standard in the IL-1 bioassay.

IL-2: Recombinant human interleukin-2 from *E.coli* (specific activity of 2×10^7 U/mg protein), cross-reactive with mouse cells, was kindly provided by Dr. Conradt, GBF, Braunschweig. In cell culture and cell activation, IL-2 was used as stimulating factor.

IL-3: Recombinant murine interleukin-3 from *E.coli* from Behring (specific activity of 5×10^7 CFU/mg protein) was used as standard in the IL-3 bioassay.

IL-6： Recombinant human interleukin-6, cross-reactive with mouse cells, from yeast from Genzyme (specific activity of 1×10^7 U/mg protein) was used as standard in the IL-6 bioassay.

IL-10： Recombinant murine interleukin-10 from COS7 from Phar Mingen (specific activity of 470 U/mg) was used as standard in the ELISA for IL-10.

GM-CSF: Recombinant murine granulocytes-macrophages colony-stimulating factor from yeast (biological activity of 5×10^7 CFU/mg protein) was kindly provided by company Behring. This factor was used as growth factor in the cell culture and as standard in the GM-CSF bioassay.

CSF-1: Recombinant human macrophage colony-stimulating factor from *E.coli* (biological activity of 1×10^7 CFU/mg protein), cross-reactive with mouse cells, also obtained as a gift from Behring, was used as a specific growth factor for the cells of macrophage system [Stanley et al. 1983].

TNF-α: Recombinant murine tumor necrose factor-α from *E.coli* (specific activity of 4×10^5 to 4×10^9 U/mg protein) from Genzyme was used as Standard in the TNF-α bioassay.

IFN-γ: Recombinant murine gamma-interferon from *E.coli* (specific activity of 1×10^7 U/mg protein) was purchased from Bender Wien (through Boehringer Ingelheim) and was used for cell activation and as standard in the ELISA for IFN-γ.

2.4. Antibodies

Anti-Cell Surface Markers

B220: 1) Rat anti-mouse B220 (CD45R, B cell marker) from Phar Mingen (Cat. No: 01121D) was used in the depletion of B lymphocytes. The dilution for use was 1：40.

2) FITC labeled rat IgG2a anti-mouse Ly5 (B220) from clone RA3-6B2 (Medac, Hamburg, Cat. No: RM2601) was used in the fluorescence staining for detection of B lymphocytes.

CD3: 1) Rat IgG2a anti-mouse CD3 (specific T cell marker) from clone KT3

(SeroTec, England Code: MCA500) was used in the depletion of T lymphocytes. The dilution for use was 1：40.

2) FITC labeled hamster IgG anti-mouse CD3 (Medac, Cat. No: HM3401) was used for studying cell phenotypes.

CD4: FITC labeled rat anti-mouse L3T4 (CD4, T helper cell marker) from clone YTS 191.1 (Medac, Cat. No: RM2401) was used for checking on the presence of T helper cells.

CD8: FITC labeled rat anti-mouse Lyt-2 (CD8, cytotoxic T cell marker) from Becton Dickinson (Order No: 1353) was used for checking on the presence of cytotoxic T cells.

F4/80: Rat IgG2b anti-mouse macrophages [Anstyn & Gordon 1981], a kind gift from Dr. Siamon Gordon (University of Oxford, UK), was provided as dialyzed ammonium sulfate precipitate from tissue culture supernatant and was used as the first antibody in indirect fluorescence staining for macrophages. The dilution for use was 1：20.

Mac-1: FITC labeled rat IgG2b anti-mouse macrophages (Mac-1, marker of myeloid cells) from clone M1/70.15 (Medac Cat. No: RM2801) was used for studying the cell surface marker.

NK-1.1: Mouse IgG2b anti-mouse NK-1.1 (marker of mouse NK precursors and mature NK cells [Hackett, et al. 1986]) from clone PK136 from American Type Culture Collection was purified by protein A-sepharose followed by

filtration via spherogel and was used in the examination of cell surface marker.

Leu-M3: Mouse IgG2b anti-human monocytes and macrophages was used as isotype control in antibody staining.

Anti-Immunoglobulins

FITC-goat $(Fab')_2$ anti-mouse IgG	Co. Dianova	Code No. 115-016-062
FITC-goat $(Fab')_2$ anti-rat IgG	Co. Dianova	Code No. 112-016-062
AP-goat $(Fab')_2$ anti-rat IgG	Co. Dianova	Code No. 112-056-062

Anti-Special Proteins and Substances

anti-IL-3:	Rat IgG1 anti-murine IL-3		
	(for neutralization)	Co. Oncogene Science	Code No. GF17
anti-IL-10:	Rat IgG1 anti-murine IL-10		
	purified	Co. Phar Mingen	Cat.No.18141D
	biotinylated	Co. Phar Mingen	Cat.No.18152D
anti-IFN-γ:	Rat IgG1 anti-murine IFN-γ		
	purified	Co. Phar Mingen	Cat.No.18181D
	biotinylated	Co. Phar Mingen	Cat.No. 18112D

anti-TNF-α: Rabbit IgG + IgM anti-murine TNF-α (for neutralization) Co. Genzym Code. IP-400

anti-perforin: mAb CB.5.4 rat anti-murine perforin, a generous gift from Prof. Tschopp, Institute for Biochemistry, University de Lausanne, Schweiz.

2.5.Enzymes

Trypsin: Trypsin (from bovine pancreas) was purchased as a 2.5% solution (for cell culture) from company Boehringer Mannheim (Order No: 210234).

Klenow Enzyme: For the cDNA labeling, Klenow enzyme was acquired (labeling degree, 2 U/μl) from company Boehringer Mannheim (Order No: 1008404) .

2.6. Isotopes

[^{3}H] Thymidine: Specific activity of 25 Ci/mmol, was obtained from IRE (Düsseldorf) and used in the [^{3}H]-Thymidine-incorporation assay for investigation of cell proliferation.

$Na_2[^{51}CrO_4]$: Specific activity of 423 mCi/mg, was acquired from NEN (Dreieich) and used in ^{51}Cr-release assay for the test of cytotoxicity.

[α-^{32}P] -dCTP: Specific activity of 3000 Ci/mmol, was purchased from Amersham-Buchler (Braunschweig) and used in the labeling of cDNA probe for Northern blot analysis.

2.7. Media

2.7.1 RPMI 1640 Medium

To 1 liter in dist. H_2O dissolved RPMI 1640 medium, 10^5 units penicillin, 0.1 g streptomycin, 15 mg phenolic red as a pH indicator and 2 g $NaHCO_3$ as a buffer substance were added, the pH value was adjusted to 6.9 with CO_2. The solution was sterilely filtered and stored at 4°C.

2.7.2 Complete Medium

The complete medium consists of 90% RPMI 1640 medium and 10% fetal calf serum (FCS) which has been heat-inactivated at 56°C for 30 min. The complete medium contains all necessary growth factors for the cells, is therefore the most commonly used medium for cell culture.

2.7.3 L Cell-Conditioned Medium

L929 fibroblast cells secrete spontaneously colony-stimulating factors (CSF), which are necessary for the proliferation and maturation of macrophages [Stanley & Heard, 1977]. To obtain the L cell-conditioned medium, 10 ml complete medium was given to 3×10^6 L929 fibroblast cells that had previously been seeded in a 9-cm tissue culture dish (Falcon 3003). This was followed by a 4-day incubation at 37°C in 5% CO_2. Afterwards, the cell free supernatant of L929 cells was collected and used as a CSF-containing medium (shortly named as “L-cond. medium” in this work, translation notes). The medium was stored at -20°C.

2.7.4 Iscove's Medium (Minimum Essential Medium Iscove’s Modification)

To 1 Liter in dist. H_2O dissolved Iscove's medium, 2 g $NaHCO_3$, 10^5 units penicillin and 0.1 g streptomycin were given. 15 mg/l phenolic red was added as pH indicator and the pH value of the mediums was adjusted to 6.9 with CO_2. After sterile filtration, the finished Iscove's medium was stored at 4°C. Iscove's medium was used for the soft agar

assay and as medium in the IL-1 bioassay.

2.8. Buffers and Solutions

2.8.1. For Cell Biological Work

2.8.1.1 PBS$^+$-MC (Phosphate-Buffered Saline):

Na_2HPO_4	8.1 mM
KH_2PO_4	1.5 mM
NaCl	140 mM
KCl	2.5 mM
Penicillin	10^5 Units/l
Streptomycin	0.1 g/l
pH 7.4	

The pH was adjusted by HCl. The solution was sterilely filtered and then stored at 4°C. For the buffer referred to as PBS-MC, penicillin and streptomycin were not added.

2.8.1.2 Actinomycin D Solution:

Actinomycin D	5 mg
70% Ethanol	0.5 ml
PBS$^+$-MC	4.5 ml

Actinomycin D was first dissolved in 70% ethanol, then filled with PBS and stored at 4°C. This solution was used for the TNF activity assay to inhibit the cell division of L929.

2.8.1.3 Glycerin-Gelatine Solution:

Gelatine	10 g
PBS-MC	70 ml

Glycerin	30 ml

At about 60°C, gelatine was dissolved in PBS-MC, the solution was cooled to hand-warm and glycerin was added. At 4°C, this jelly was unlimitedly durable. Before use, the jelly was heated at 60°C and the air-dried cytopreparations were covered with the glycerin-gelatine solution cooled to 40°C.

2.8.1.4 Buffered Formalin-Aceton Solution:

$Na_2HPO_4 2H_2O$	40 mg
KH_2PO_4	200 mg
dist. H_20	100 ml
Aceton	90 ml
Formalin	50 ml

The substances were mixed in the order indicated above. The prepared solution showed a pH value of ca. 6.6 and was used for cell fixation in the double esterase staining.

2.8.1.5 2-ME/Hepes Stock Solution:

2-Mercaptoethanol (2-ME)	10 µl
H_2O	10 ml
1 M Hepes Solution	12.5 ml
Complete medium	27.5 ml

2-Mercaptoethanol was first mixed with H_2O, sterilely filtered, then mixed with the sterile Hepes solution and complete medium. As a result, the produced stock solution contains approximately 3 mM 2-mercaptolethanol and 0.25 M Hepes, and was stored in aliquots at -20°C.

2.8.1.6 MTT Solution:

MTT	50 mg
PBS^+-MC	10 ml

MTT was dissolved in PBS^+-MC, sterilely filtered and stored at 4°C. The MTT solution was used in the colorimetric bioassay (MTT test).

2.8.1.7 MTT-Stop Solution:

N, N-Dimethylformamide	200 ml
dist. H_2O	200 ml
SDS	40 g

In a 500 ml glass bottle, DMF (N, N-Dimethylformamide) was first mixed with H_2O. Then SDS was given into the bottle. At 37°C, the bottle was left to stand overnight to dissolve the SDS. The solution was kept at 37°C.

2.8.1.8 Percoll Stock Solution (60.6%):

Percoll	60 ml
3 × RPML 1640	30 ml
FCS	9.8 ml

This solution served as stock solution to build up the Percoll gradients and could be stored at 4°C for several months. The osmolarity of the solution stands at 285 m OSM/Kg H_2O.

2.8.1.9 Buffer for the Esterase-Double Staining:

Stock solution A: 10 ml 1M KH_2PO_4 + 140 ml H_2O

Stock solution B: 10 ml 1M Na_2HPO_4 + 140 ml H_2O

Sorensen buffer:

pH 6.3 77.35 ml stock solution A + 22.65 ml stock solution B

pH 7.4 19.60 ml stock solution A + 80.40 ml stock solution B

1) For the Chloroacetate Esterase Staining:

Sorensen-Buffer pH 7.4	190 ml
Fast Blue BB Salt	200 mg
Naphthol AS-D Chloroacetate	20 mg
DMF	10 ml

In a glass test tube, naphthol AS-D chloroacetate was dissolved in DMF (N, N-dimethylformamide) and added to the buffer/dye mixture. The mixture was always freshly prepared, protected from light and filtered through Whatman-filter paper (No.54) immediately before the staining.

2) For 2-Naphtyl butyrate Esterase Staining:

Sorensen-Buffer pH 6.3	190 ml
Fast Garnet GBC	200 mg
α-Naphthyl butyrate	200 µg
Ethylene glycol monomethyl ether	8 ml

α-Naphthyl butyrate was mixed with ethylene glycol monomethyl ether and added to the buffer/dye mixture. Before each use, it was freshly prepared and protected from light.

2.8.1.10 Trypsin/EDTA Solution:

NaCl	8 g
KCl	0.4 g
α-D-Glucose	1.0 g
$NaHCO_3$	0.35g
EDTA	0.2 g

The substances were dissolved one after the other in 1 liter of dist. H_2O and

mixed with 20 ml 2.5% trypsin solution, thus a final concentration of 0.05% (w/v) trypsin was created in the solution. After sterile filtration, the solution was frozen in aliquots at -20°C for storage.

2.8.2. For ELISA, FACS and Histochemical Stainings

2.8.2.1 TBS Buffer:

Tris-HCl	100 mM
NaCl	150 mM
pH 7.5	

2.8.2.2 DAB-H_2O_2 Solution:

DAB	7 mg
TBS	10 ml
H_2O_2	4 µl

DAB (3,3'-Diaminobenzidin-Tretrahydrochlorid, substrate for peroxidase) was dissolved in TBS. Shortly before use, H_2O_2 was added. For storage, the DAB/TBS solution could be frozen at -20°C, then thawed and supplemented with H_2O_2 prior to use.

2.8.2.3 ELISA Wash Buffer:

NaCl	0.3 M
Tween 20	0.1%

Dissolved in PBS-MC and stored at 4°C.

2.8.2.4 FACS Wash Buffer:

FCS	2%
NaN_3	0.01% (w/v)

Mixed in PBS^{+}-MC, stored at 4°C.

2.8.2.5 Fast Red-Substrate Solution:

Naphthol AS-MX Phosphate	2 mg
N, N-Dimethyl Formamide	0.2 ml
0. 1 M Tris-Buffer pH 8.2	9.8 ml
1 M Levamisole	0.01 ml
Fast Red TR	10 mg

First, naphthol AS-MX phosphate was dissolved in N, N-dimethyl formamide (in glass test tube), then Tris-buffer and levamisole were added. This solution is durable at 4°C for several weeks and longer at -20°C. Shortly before staining, Fast Red TR (substrate for alkaline phosphatase [Dejong et al. 1985]) was dissolved in it and given on the slides through a filter (0.22 μm, Millipore, No: SLGS 025BS).

2.8.2.6 TMS Buffer (Alkaline Phosphatase Buffer):

Tris-HCl	100 mM
NaCl	100 mM
$MgCl_2$	50 mM
pH 9.5	

2.8.3. <u>For the Molecular Biological Work</u>

2.8.3.1 20 × SSC Solution:

NaCl	3 M
Na-Citrate	0.3 M
pH 7.0 (20°C)	

Prepared with dist.H_2O, autoclaved and then stored at RT.

2.8.3.2 50 × Denhardt's Solution:

Ficoll	1 %
Polyvinylpyrrolidone	1%
BSA	1%

in H_2O bidist.

Sterilely filtered and stored in aliquots at -20°C.

2.8.3.3 DEPC-H_2O:

H_2O bidist. + 0.1% DEPC

Leave to stand overnight, then autoclave.

2.8.3.4 DMSO-Mix:

H_2O	150 µl
Dimethyl sulfoxide	1750 µl
0.5 M Na-phosphate buffer (pH 7)	70 µl
20% SDS	35 µl

The mixture was stored in 150 µl aliquots at -20°C.

2.8.3.5 Hybridization Buffer for Northern Blot Analysis:

SSC	5 ×
Denhardt′s Solution	5 ×
SDS	0.5%
EDTA	10 mM

Kept at RT.

2.8.3.6 Hybridization Buffer for *in situ* Hybridization:

SSC	4 ×

Formamide	50%
Denhart's Solution	1 ×
Dextran Sulfate	5%
Salmon Sperm DNA	0.5 mg/ml
Yeast tRNA	0.25 mg/ml

Prior to hybridization, the buffer was freshly prepared from the stock solutions (2 × SSC, 50 × Denhart's, 28% dextran sulfate, 10 mg/ml salmon sperm DNA and 5 mg/ml Yeast tRNA).

2.8.3.7 5 × RNA Sample Buffer:

Na-phosphate buffer (pH 7)	10 mM
Ficoll	10%
Brome phenol blue	0.4%

The buffer was prepared with water, autoclaved and stored at -20°C.

2.8.3.8 Solution D:

Guanidinium isothiocyanate	4 M
Sodium citrate	25 mM (pH 7)
Sarcosyl	0.5%
2-Mercaptoethanol	0.1 M

To minimize the handling with guanidinium isothiocyanate (GTC), 250 g GTC in the original packaging was dissolved with 293 ml water, 17.6 ml 1 M sodium citrate (pH 7) and 26.4 ml 10% sarcosyl at 65°C. This stock solution could be kept at RT for at least 3 months. Solution D was then prepared by adding 72 µl 2-mercaptoethanol into 10 ml stock solution. This solution was stored at 4°C and used for RNA isolation [Chomczynski & Sacchi 1987].

2.8.3.9 10 × TE Buffer:

Tris-HCl	100 mM
EDTA	10 mM
pH 8.0	

Prepared with H_2O bidist., autoclaved and kept at RT.

2.9. Cell Lines

2.9.1 D10.G4.1

D10.G4.1 is a cloned mouse T helper cell line [origin: AKR/J(H-2k) mice]. In absence of IL-1, this cell line grows only minimal after Con A stimulation. Therefore, it was used as a trustworthy and sensitive cell line for bioassay on IL-1 [Kaye et al. 1983]. The D10.G4.1 cells are cultivated in Iscove's medium with 5% FCS, 1 mM sodium pyruvate, 1% 2-ME/Hepes stock solution, and ca. 7% supernatant from Con A-stimulated rat spleen cells in 6-well microtiter plates (Nunc, Denmark).

2.9.2 FDCP-1

FDCP-1 is a murine factor-dependent hematopoietic precursor cell line [Naparstek et al., 1986] and proliferates only in response to GM-CSF or IL-3 [Magee & Wing, 1989]. In the present work, FDCP-1 cells were cultured in complete medium plus ca. 10% IL-3-containing medium (Wehi-3D cell supernatant) in tissue culture dishes (Falcon 3003). They were used to determine GM-CSF- or IL-3-activity in supernatants.

2.9.3 L929

The adherent L929 fibroblast line (origin: C3H mice) was cultured in complete medium in tissue culture dishes (Falcon 3003). To detach the cells, the medium was removed, 3 ml trypsin/EDTA solution (2.8.1.10) was added on the L929 cell-turf and incubated at RT for 4-5 min. Then the trypsin/EDTA solution was carefully removed, the cells were

flushed with complete medium and washed twice. The washed L929 cells were resuspended in complete medium, diluted and reseeded in culture dishes. L929 cells were used as TNF-sensitive tumor cells for the TNF activity test in this work.

2.9.4 P815

P815 Mastocytoma cells (origin: DBA/2J mice, methylcholanthrene-induced) were maintained in complete medium as suspension culture in Greiner petri dishes. This tumor cell line is NK-resistant, but can be lysed by LAK cells and activated macrophages. For determination of NK- or LAK-activity, P815 were used either as control or as target cells in the cytotoxicity test.

2.9.5 7TD1

The hybridoma cell line 7TD1 (fusion of mouse myeloma cell line SP2/0-AG14 with spleen cells from a C57BL/6 mouse immunized with 50 µg *E.coli* LPS) grows only in the presence of IL-6 and is therefore used for bioassay on IL-6 [Van-Snick et al. 1986]. For culture, the cells are maintained in complete medium supplemented with 2% 2-ME/Hepes stock solution (2.8.1.5) and 5-10% IL-6 containing medium (3T3 cell supernatant) in 50 ml tissue culture bottles (Nunc, Denmark 1 63371).

2.9.6 Yac-1

Yac-1 is an induced lymphoma cell line by inoculation with Moloney leukemia virus (MLV) in a neonatal A/Sn mouse. This cell line is very sensitive to the cytotoxic activity of NK cells from mouse [Kiessling et al. 1975 a and b]. Yac-1 was used as target cells for the NK activity test and maintained *in vitro* with complete medium in 9-cm tissue culture dishes (Falcon 3003).

Methods

2.10. Cell-Isolation, -Culture, -Purification and -Activation

2.10.2 Isolation of Bone Marrow Cells

Mouse femurs were aseptically removed, cleaned and cut open at the ends. The bone marrow was flushed out with the help of a syringe and a 27G (0.4-mm in diameter) needle, then suspended in RPMI 1640 medium. After one time washing, the bone marrow cells were resuspended with complete medium at a concentration of 3×10^7 cells/ml. The cell suspension was carefully placed on the top of a 4-layer Percoll gradient (gradient I: 32. 0%, 52.0%, 55.0%, 60.6%) and centrifuged at 1500 rpm (425 *g*) for 30 min. After centrifugation, the cell fraction between Percoll-layer 1 (32,0%) and -layer 2 (52,0%) was collected, washed three times and seeded for bone marrow pre-culture (see 2.10.3).

2.10.2 Percoll Gradient Fractioning

Shortly before building up a gradient, the Percoll stock solution (60.6%, see 2.8.1.8) was diluted with complete medium for the desired concentrations as shown below. Each 2 ml of finished diluted Percoll solutions was carefully stepwise filled in the conical centrifuge tubes (Falcon 2099). $3\text{-}6 \times 10^7$ cells in 2 ml complete medium were placed on the surface of the gradient using a 2 ml pipette. The gradients were centrifuged for 30 min (gradient I at 425 *g* and gradient II at 550 *g*). The resulting separated cell fractions were collected with the help of a syringe and a 20 G (0.9 × 70 mm) needle and washed three times with RPMI 1640.

Preparation of Percoll Layers for Gradient I:

Layer	Volume	Percoll Stock Solution	Complete Medium
1 (32.0%)	4.5 ml	2.376 ml	2.124 ml
2 (52.0%)	4.5 ml	3.821 ml	0.579 ml
3 (55.0%)	4.5 ml	4.125 ml	0.375 ml
4 (60.0%)	4.5 ml	4.500 ml	0.000 ml

Preparation of Percoll Layers for Gradient II:

Layer	Volume	Percoll Stock Solution	Complete Medium
1 (32.0%)	4.5 ml	2.376 ml	2.124 ml
2 (40.8%)	4.5 ml	3.063 ml	1.437 ml
3 (45.3%)	4.5 ml	3.398 ml	1.103 ml
4 (50.0%)	4.5 ml	3.750 ml	0.750 ml
5 (55.0%)	4.5 ml	4.125 ml	0.375 ml
6 (60.6%)	4.5 ml	4.500 ml	0.000 ml

2.10.3 Bone Marrow (BM) Pre-Culture

$10 \times l0^6$ cells (2.10.1) were seeded in 15 ml complete medium in 9-cm tissue culture dishes (Greiner). For the expanding of macrophage precursors, the culture was supplemented with 10-15% CSF-containing L cell-conditioned medium (2.7.3). For some investigations (for example: Northern blot analysis, cytokine production), instead of L-cond. medium, the recombinant GM-CSF (200 U/ml) was used, in order to avoid the possible unknow factors existing in L-cond. medium to act on the cells. In low concentration (< 200 U/ml), GM-CSF acts mainly on the proliferation of the cells of macrophage system [Metcalf, 1987]. For generation of NK activity, IL-2 (100 U/ml) was added to the BM pre-culture. For this purpose, the complete medium with 5 μM 2-ME [Kuppen et al. 1991] was used. The BM pre-culture always ran only for 3 days.

2.10.4 Isolation of Macrophage Precursors from BM Pre-Culture

After a 3-day BM pre-culture, the nonadherent cells were collected, washed, and fractionated via a 6-layer Percoll gradient II (32.0%, 40.8%, 45.3%, 50.0%, 55.0% and 60.6%). The cell fraction between Percoll-layer 2 (40.8%) and -layer 3 (45.3%) was collected and washed three times with RPMI 1640. This cell fraction contained approximately 85% macrophage precursors (including some slightly adherent macrophages), about 10% polymorphonuclear cells, and 2-5% lymphocytes.

2.10.5 Depletion of Phagocytic Cells

The cells were suspended in complete medium to 1×10^6 cells/ml and incubated with iron particles (Technicon Instruments Corp. Tany town, NY. Product No: T01-0507) in a plastic dish (Falcon 3003) for 30 min at 37°C. During the incubation, the phagocytic cells ate iron particles. After the incubation, the nonadherent cells were collected and transferred into a 10 cm long tube (Falcon 2057). The tube was put in a Dynal Magnet (Dynal MPC-1, "magnetic particle concentrator", Dynal, Oslo, Prod. No. 12001) to remove the nonadherent but iron-particle-containing (i.e. phagocytic) cells. After the phagocytic cells were firmly attached to the wall of the tube, the cell suspension in the tube was suctioned out of the tube using a syringe and a 20 G (0.9 × 70 mm) needle. The obtained cell population was free of phagocytic and adherent granulocytes and semi-mature macrophages.

2.10.6 Depletion of Lymphocytes with Magnetic Beads

First, the cells were incubated with antibodies anti specific surface markers of unwanted lymphocytes (rat anti-mouse CD3 for T lymphocytes and rat anti-mouse B220 for B lymphocytes) at 4°C for 45 minutes. After the incubation, the cells were washed three times with PBS, resuspended with PBS + 2% FCS at 2×10^6 cells/ml and

transferred into 10 ml Falcon test tube. Magnetic beads from company Dynal, coated with sheep anti-rat IgG, were given at a ratio of 4 : 1 (beads to cells) into the cell suspension. The tube was incubated at 4℃ for 30 min on a rotary wheel. Afterwards, the magnetic beads and the cells that bound on the beads through antibodies were removed by Dynal Magnet MPC-1. The remaining cell suspension was centrifuged and the cells were washed twice. Thereby the purified cell population contained less than 0.5% lymphocytes.

2.10.7 Main-Culture and LAK-Culture

The macrophage precursor cells isolated from 3-day BM pre-culture (2.10.3) were further cultivated as the starting population in complete medium with 2-ME (5 μM). Depending on the purpose of the study, corresponding factors (CSF-1 or IL-2, or CSF-1 + IL-2) were added into the culture in different concentrations. This culture was referred to as the main-culture. For LAK-culture, the main-culture was given with high concentration of IL-2 (1000 U/ml).

2.10.8 Activation of BM Macrophage Precursors for Cytokine Production

To investigate the cytokine production capacity of bone marrow macrophage precursor cells, the washed cells were incubated overnight in complete medium (0.5×10^6 cells/ml) with corresponding stimulating factors at 37℃. Supernatants were collected next day (about 20 hours after the factors were added) and stored in polypropylene tubes (Falcon 2063) at 4℃. Cytokines were detected either in bioassay (2.12.4) or in ELISA (2.12.5).

2.11. Cell Staining

2.11.1 Cytopreparations

For each microscopic slide, 1×10^5 cells were spun on it in a cytocentrifuge (Cytospin,

Shandon Southern, Astmoor, GB) for 5 min at 500 rpm, and then air-dried. The cytopreparations made in this way can be used by various staining methods for studying cell morphology, cell surface markers and cytoplasmatic components. For storage, the dried cytopreparations can be wrapped separately from each other in aluminum foil and frozen at -20°C.

2.11.2 Cell Culture in Chamber Slides

Cells were suspended in complete medium with 0.2% 2-ME/Hepes stock solution (2.8.1.5) at a cell concentration of 2.5-5 × 10^5 cells/ml and 300 µl per well was seeded in the 8-well chamber slides (Nunc, IL. order No.: 177402). The corresponding supplements such as PMA, M-CSF, and IL-2 were added into the culture depending on the purpose of the study. The slides were incubated at 37°C in 5% CO_2.

2.11.3 Esterase Double Staining

The cytopreparations were first fixed in the buffered formalin-acetone solution (see 2.8.1.4) for 30 sec. at 4-10°C, then flushed 3 min with PBS, and afterwards air dried. The fixed cytopreparations were incubated for 15 min in dark with the staining-solution (described under 2.8.1.9) for Chloroacetate esterase (specific for granulocytes) at RT, and then flushed 3 min with dist. H_2O. After the cytopreparations were air dried, they were stained in dark for another 25-30 min in the filtered staining-solution for α-Naphthyl butyrate esterase (see 2.8.1.9) to detect monocytes and macrophages (positive for α-Naphthyl butyrate esterase). Afterwards, the cytopreparations were flushed 3 min with H_2O and then air dried. They were counter-stained with Mayer's Hemalaun-solution (for nucleus staining) for about 4 min. After thoroughly flushed with water and then air dried, the cytopreparations were covered with the glycerin-gelatine solution (2.8.1.3).

2.11.4 Fluorescence Staining of Cell Surface Markers

1-5 × 10^5 cells in 40 µl FACS wash buffer (2.8.2.4) were suspended in 0.6 ml tubes (Greiner 101101). The cells were first mixed with 40 µl human Ig solution (10 µg/ml) and incubated for 15 min on ice to block the unspecific binding on Fc receptors on the cell surface. After one time washing, the cells were incubated either in 40 µl FACS wash buffer alone or with 40 µl antibody solution (antibody diluted in FACS wash buffer) for 45 min on ice. It was followed by three time washing with the wash buffer. Subsequently, the FITC-conjugated second antibody was added and further incubated on ice. After 30 min incubation, the cells were again washed three times and were finally analyzed on a flow cytometer (FACScan Becton, Dickinson GmbH, Heidelberg). For direct fluorescence staining, immediately after the blocking step the cells were incubated with FITC-antibody on ice for 30 min.

2.11.5 Immunohistochemical Staining with AP-Conjugated Antibody

Fresh or frozen cytopreparations were fixed in 100% isopropanol at RT for 10 min. After two time washing with TBS + 0.1% Tween 20, they were preincubated with goat serum (1：10 diluted in TBS) for 40 min. After the goat serum had been removed (washed one time with TBS, translation notes), the cytopreparations were incubated with antibody solution (rat anti-murine perforin 1：400 diluted in TBS) in a humid chamber at RT for 45 min. This was followed by two time washing with TBS + Tween 20. As the next step, 1：20 diluted goat anti-rat IgG (conjugated with alkaline phosphatase) was given on the cytopreparations and incubated in humid chamber at RT for 45 min. After the incubation, the cytopreparations were first flushed three times with TBS + Tween 20, then Fast Red-substrate solution (2.8.2.5) was added through a filter (0.22 µm, Millipore SLGS025BS) on them and left for 10-30 min reaction. By flushing with TBS, the color development was stopped. The subsequent counter-staining (nucleus staining) was made by shortly inserting cytopreparations in hematoxylin (only for 5-10 sec.), then immediately flushed with water. The finished

stained cytopreparations were coated with Aquatex.

2.11.6 Pappenheim Staining (May-Greenwald/Giemsa-Staining)

Cytopreparations were coated with May-Greenwald-Solution for 3 min and thereby fixed and stained (the acidic components of the cells can be stained bluish during this treatment). After a short wash in distilled water, they were counter-stained with the Giemsa-Solution（1：10 diluted in distilled water）for 10 min. In this step, the alkaline acidophilic proteins were stained red with the acidic dye. After the cytopreparations were thoroughly washed with distilled water and then air dried, they were ready for the light microscopic investigation.

May-Greenwald-Solution: 0.25% (w/v) May-Greenwald in methanol

Giemsa-Solution: 0.4% (w/v) modified Giemsa dissolved in buffered methanol (pH 6.8).

2.12. Tests

2.12.1 [^{3}H]-Thymidine Incorporation for the Test of Cell Proliferation

The cells to be tested were seeded into 96-well microtiter plates (Falcon 3072) at 5×10^3 cells per well in 200 µl complete medium and incubated with or without growth factors at 37°C in 5% CO_2 for different time periods. 1 µCi per well of [^{3}H]-Thymidine was then added into the plates and incubated for further 18 hours. The cells in the plates were finally harvested with a cell harvester (Skatron, Norway) on glass fiber filters (Schleicher & Schüll, Dassel). The dried filter platelets were laid in plastic tubes (Scintillation Vials & Caps, Skatron, Cat. No. 15765) and mixed with 3 ml scintillation fluid. The radio activity absorbed by the cells was measured in a liquid scintillation counter (Beckman LS1801). The radio activity is expressed in counts per minute (cpm).

2.12.2 ^{51}Cr Release from Target Cells for the Test of Cytotoxicity

First, the target cells were labeled with $Na_2{}^{51}CrO_4$ (200 μCi/5 × 10^6 cells) in 0.5 ml complete medium for 1.5 hours at 37℃ in 5% CO_2. After the labeling, the target cells were washed three times with RPMI 1640 to remove extracellular ^{51}Cr, and then resuspended with complete medium at 1 × 10^5 cells/ml. For the test of cytotoxicity, 100 μl effector cell suspension with different cell number (4 × 10^5, 2 × 10^5, 1 × 10^5, 5 × 10^4, 2 × 10^4 and 1 × 10^4 cells) per well were plated in round-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark). Into these plates, the radioactive labeled target cells (1 × 10^4/well) were pipetted. The resulting ratios of effector and target cells were: 40：1； 20：1； 10：1； 5：1； 2：1 and 1：1. The microtiter plates were shortly centrifuged at 600 rpm and incubated at 37℃ in 5% CO_2. After 4 hours (for testing NK activity) or 16 hours (for testing LAK activity) lysis time, the microtiter plates were centrifuged for 10 min at 1200 rpm, 100 μl aliquots of the supernatants were collected and the radio activities contained in them ("Experimental Release": ExpR) were measured in a gamma ray measuring device (Kontron MR 480, Basel, Schweiz). For controls, the spontaneous ^{51}Cr-release from the target cells (without presence of effector cells, referred to as SR) and the maximum ^{51}Cr-release (100% killing of target cells by incubation with 1N HC1: MaxR) were determined. The cytotoxicity of effector cells was expressed either as % specific lysis of target cells, calculated according to the formula below, or lytic units (LU):

$$\% \text{ Specific Lysis} = \frac{\text{ExpR - SR}}{\text{MaxR - SR}} \times 100$$

When the results were expressed as LU, one lytic unit (1 LU) represents the number of effector cells required to cause 20% specific lysis of 1 × 10^4 target cells. LU were

calculated from different ratios of effector- and target-cells. LU/10^7 indicates the lytic units of 1×10^7 effector cells.

2.12.3 Test of Calcium-Dependence of Cytotoxicity

To determine whether the cytotoxicity of the cells was dependent on the presence of calcium, three test media were used: A) complete medium, B) complete medium + 10 mM EDTA and C) complete medium + 10 mM EDTA + 15 mM $CaCl_2$. EDTA can form complexes with the calcium ions contained in the complete medium. As a result, Ca^{2+} was depleted in medium B. In medium C, $CaCl_2$ was added in excess so that it contained Ca^{2+} again. The effector cells to be tested were divided into three portions (washed three times with calcium-free PBS, translation notes), suspended in the corresponding test media, and plated in 96-well microtiter plates. Cytotoxicity tests were performed as described under 2.12.2.

2.12.4 MTT Test (Colorimetric Bioassay)

Since only living cells are capable of converting MTT (formation of blue formazan crystals), MTT test is often used to determine cell growth. This test is also called Colorimetrical bioassay [Mosmann 1983; Tada et al. 1986]. In the present work, the MTT test was performed for investigations of cytokine activities in cell supernatants. The cytokine-dependent cell lines were cultivated in 96-well microtiter plates with the supernatants to be tested as well as appropriate cytokine standards. For the controls, the cells were incubated with medium without factor. After the cells had died in control-wells (ca. 2-4 days), 10 µl MTT solution (2.8.1.6) per well was pipetted into the plates. After about 4-hour incubation at 37°C, 100 µl MTT-stop solution (2.8.1.7) were given per well. The plates were left to stand overnight at 37°C to achieve a complete dissolution of the blue crystals formed. A photometric absorption measurement was performed by a Microplate Reader (MR 700 Dynatech) at 570 nm.

2.12.5 Enzyme-Linked Immunosorbent Assay (ELISA)

In this work, the ELISA was mainly used to study the cytokine content in supernatants. First, the plates (Nunc Immunoplate I, Code No.439454) were coated with appropriate anti-cytokine "capture antibodies" 50 µl per well (2.5 µg/ml) and then incubated overnight at 4°C. After washing twice with ELISA wash buffer (2.8.2.3), 100 µl PBS + 0.5% BSA was added per well to block the plastic surface and the plates were incubated for one hour in a humid chamber at RT. It was followed by two washes and then the supernatants to be tested as well as cytokine standard solutions were pipetted in (50 µl/well). The plates were incubated in a humid chamber for 2 hours at RT, then washed five times. As next step, an incubation of the plates with 1：100 diluted biotinylated antibody anti the same cytokine (50 µl/well) was performed in a humid chamber at RT for 1 hour. Afterwards, the plates were washed six times, and incubated with 1：500 diluted Avidin-POD-conjugate (50 µl/well) in a humid chamber at RT for 30-45 min. In the end, they were washed again six times. Subsequently the DAB-H_2O_2 solution (see 2.8.2.2) was filled into the plates at 50 µl per well and the plates were then left in dark for 10-20 min at RT. The color reaction was stopped by adding 100 µl 1N HCl per well. The results were evaluated using the Microplate Reader.

2.12.6 Soft-Agar Assay

30-60 × $l0^6$ fresh bone marrow cells from C57BL/6 mice aged 4 weeks were suspended in 3 ml PBS, then carefully layered on 5 ml Ficoll and centrifuged at 2500 rpm. After centrifugation, the cell fraction between PBS and Ficoll was collected and washed three times. The cells were then resuspended in the Iscove's medium mixed with 15% FCS at 1 × 10^5 cells/ml. Each 0.1 ml of supernatants to be tested or CSF-containing solutions was filled into 3.5 cm tissue culture dishes (35 × 10 w/grid, Nunc, Inc., IL. order No.:174926). The BM cell suspension was mixed in the ratio of 8：1 with 3.5% agar in 42°C water bath and then poured into the dishes (0.9 ml/dish). It was followed by a

quick mixing to evenly distribute the supernatants to be tested or CSF-solutions with the cells as well as the agar in the culture dishes. The dishes were incubated in humid atmosphere at 37°C in 5% CO_2 for 7 days. After that, the colonies that had grown up on the agar were counted under the light microscope.

To determine the cell types in the colonies, the discs of "Soft-Agar" in PBS-MC bath were placed on microscopic slides and then air dried. Subsequently, the cytopreparations were stained according to esterase double staining (2.11.3) and microscopically investigated.

2.13. Detection of Cytokines

2.13.1 IL-1

The supernatants to be tested as well as standard IL-1 (0.1-125 units/ml) were first filled into the wells of 96-well microtiter plates (Falcon 3072). The washed D10.G4.1 cells (2.9.1) were resuspended in IL-1-testmedium (Iscove's medium + 1 mM sodium pyruvate + 1% 2-ME/Hepes stock solution + 5 µg/ml Con A + 5% FCS) at 2×10^5 cells/ml, pipetted 100 µl per well into the microtiter plates, then incubated at 37°C in 5% CO_2. For the controls, D10.G4.1 cells were incubated with IL-1-testmedium alone. After appropriate incubation period, the MTT test was performed (2.12.4) and the growth of D10.G4.1 cells was determined. With the help of the IL-1 standard solutions, a calibration curve was created. The IL-1 activities of samples were read from the calibration curve.

2.13.2 IL-6

7TD1 cells (2.9.5) in complete medium + 2% 2-ME/Hepes stock solution (thus, the final concentration of 2-ME in the medium is approximately 50 µM) were pipetted into the 96-well microtiter plates ($2.5 \times l0^3$ cells per well), which had been previously coated with diluted supernatants to be tested and standard IL-6, and incubated at 37°C in 5%

CO_2 for 3-4 days. Subsequently, the MTT test was conducted. To establish the calibration curve, standard IL-6 was stepwise diluted from 125 U/ml to 0.5 U/ml in the test wells.

2.13.3 IL-10

The IL-10 content in the cell supernatants was detected by means of ELISA (2.12.5). For this purpose, the two specific anti-mouse IL-10 antibodies (purified and biotinylated, see page 18) were used in a dilution of 1：500.

2.13.4 M-CSF

To determine the M-CSF activity in cell supernatants, the soft-agar assay (2.12.6) was performed, followed by esterase double staining (2.11.3).

2.13.5 GM-CSF and IL-3

FDCP-1 cells need either GM-CSF or IL-3 in the culture medium to survive and proliferate (2.9.2). To investigate GM-CSF- or IL-3-activity of the cell supernatants, specific, monoclonal anti-mouse IL-3 antibody (see page 18) was used in the bioassay with FDCP-1 cells, in which two approaches, with and without anti-IL-3, were parallelly performed. In the investigation, FDCP-1 cells were first suspended in complete medium ($4 \times l0^5$ cells/ml) and then seeded 2×10^4 cells per well in 96-well microtiter plates that contained serially diluted standard GM-CSF (0.25-125 U/ml) or IL-3 (0.01-10 U/ml) or the supernatants to be tested. Then 10 µl anti-IL-3 antibody was pipetted into the appropriate test wells. The results of the bioassay were also evaluated by the MTT test as described above. The units of GM-CSF or IL-3 activity were calculated using the calibration curve of standard GM-CSF or IL-3 respectively.

2.13.6 IFN-γ

An ELISA (2.12.5) for detection of gamma-IFN in cell supernatants was performed with two specific anti-mouse IFN-γ antibodies (purified and biotinylated, see page 18). The antibodies were diluted 1：500.

2.13.7 TNF-α

The TNF activity in cell supernatants was determined using L929 cells. In the presence of actinomycin D, L929 cells are very sensitive to TNF mediated cytotoxicity and can be lysed within 24 hours [Ruff & Gifford 1980]. For the test, L929 cells in complete medium were seeded (1.5×10^4 cells per well) in the 96-well microtiter plates and incubated overnight at 37°C in 5%CO_2, so that a closed cell turf was formed. On these cells, serial dilutions (1：2; 1：4; 1：8 etc.) of the cell supernatants to be tested as well as the standard TNFs (0.10-200 U/ml) were prepared. In each well, 100 µl actinomycin D solution (2.8.1.2), 1：500 diluted in PRMI 1640, was added. After about 20 h incubation at 37°C in 5% CO_2, 100 µl medium was taken from each well and 10 µl MTT solution was pipetted into the well to determine the degree of the killed L929 cells. Since the actinomycin-treated cells convert MTT slower than normal cells, the incubation time was prolonged from about 4 h to 6 h. The results were evaluated as described by the MTT test (2.12.4). To determine the TNF type (TNF-α or TNF-β), specific anti-TNF-α antibody (see page 18) was used in a dilution of 1：100 in the test.

2.14. Detection of mRNA of Special Proteins

2.14.1. Northern Blot Analysis

2.14.1.1 Labeling of a cDNA Probe with [^{32}P]

For the labeling, the "Megaprimer"-Kit of company Amersham was used. The labeling was performed according to the "Random Primer" Method [Feinberg & Vogelstein1983]

as follow: 20 ng double stranded cDNA was mixed with 5 µl primer solution (nonadeoxy nucleotide in watery solution), and the mixture was filled with H_2O bidist. up to 50 µl. The DNA was denatured at 95°C for 5 min and cooled down at RT for primer attachment. After addition of 10 µl 5 × labeling buffer (dATP, dGTP, dTTP in Tris-HCl pH 7.8), 34 µl H_2O, 5 µl [α-^{32}P] dCTP (specific activity: 3000 Ci/mM) and 2 µl Klenow-enzyme (1 Unit/µl), the labeling was performed at 37°C for 30 min. Subsequently, the DNA was separated from the free nucleotides by ethanol precipitation and taken up in TE buffer (2.8.3.9). It was achieved a specific activity of > 10^9 cpm/µg DNA.

2.14.1.2 RNA Preparation

3-10 × 10^6 cells from tissue culture were centrifuged and washed twice with 5 ml ice-cold PBS. After the PBS was removed, the cell pellet was mixed with 900 µl solution D (2.8.3.8) so that the cells were immediately lysed [Chomczynski & Sacchi 1987]. The cell lysate was transferred into a 2 ml Eppendorf tube and 90 µl 2 M NaAc (pH 4.0), 900 µ1 phenol (previously saturated with water), and 180 µ1 chloroform/isoamyl alcohol (49：1) were added (after adding each individual component, it was carefully mixed). The suspension was then shaken vigorously on the vortex for 10 seconds. After cooling on ice for 15 min, the sample was centrifuged for 20 minutes at 10000 *g* in an Eppendorf centrifuge at 4°C. After centrifugation, the RNA was in the aqueous phase, DNA and protein, however, in the interphase and the phenol phase. The aqueous phase was transferred into a new 2 ml Eppendorf tube, mixed with 900 µl isopropanol and then left to stand at -20°C for at least 1 hour to precipitate the RNA that could be finally obtained by centrifugation (10 min at 10000 *g*). The RNA was resuspended in 75% pre-cooled ethanol and centrifuged at 10000 *g* for 5 min. The supernatant was carefully taken out with a curved Pasteur pipette and the precipitation was air dried for 10-15 min at RT until no more traces of ethanol were visible. The RNA was taken up in 50 µl 0.5% SDS and heated at 65°C for 15 min to dissolve it. To determine the nucleic acid

content of the sample, the extinction of the sample was measured at 260 nm. The RNA solution was either directly analyzed in the Northern blot analysis or stored at -70°C.

2.14.1.3 RNA Separation and Blot

Each 10 µl RNA solution (ca. 20 µg RNA) was mixed with 8 µl DMSO mix (2.8.3.4) and 1.2 µl glyoxal (20% in H_2O pH ca.6) and incubated for 30 min at 50°C. The samples were quickly cooled at 4°C, mixed with 4 µl 5 × RNA sample buffer (2.8.3.7), applied to a gel (1% agarose in 10 mM Na-phosphate buffer pH 7) and separated at 70 V in the same buffer. The gel was then stained with 1 µg/ml ethidium bromide in 10 mM Na-phosphate buffer for 10 min to make the RNA visible. In the end, a Northern blot was made according to the standard technique [Maniatis et al. 1982], namely: a membrane (Hybond-N^+ nylon membrane, Amersham) was placed on the gel and a capillary blot was built up, as shown in the **Figure** below. The transfer of RNA from the gel to the membrane was carried out overnight, where 2 × SSC was used as transferring buffer.

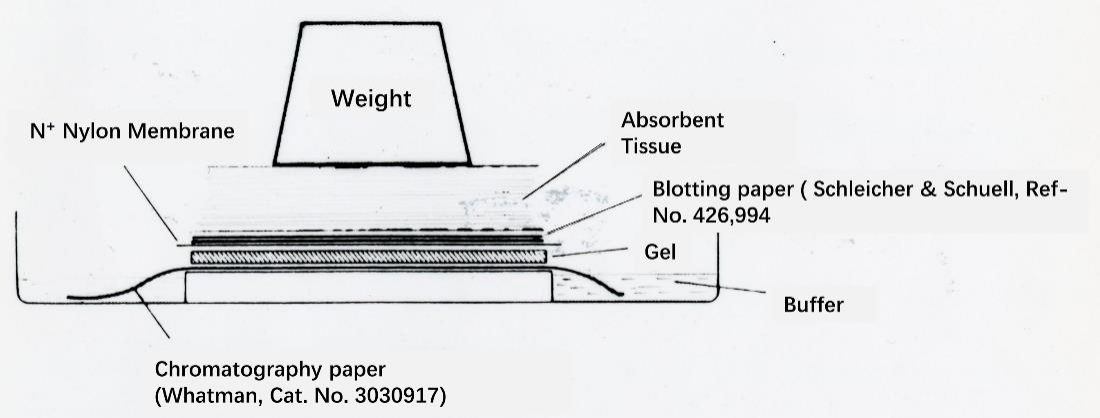


Figure. Device for a Northern-Blot

2.14.1.4 Hybridization and Detection of RNA

The nylon membrane was baked at 80℃ for 30-60 min to fix the RNA. The membrane was moistened in 2 × SSC and pre-hybridized with denatured salmon semen DNA (100 μg/ml in hybridization buffer) for 1-3 hours at 42℃. The radiolabeled cDNA denatured by heating and cooling was given into the pre-hybridization mixture and hybridized with the RNA for 12-15 h at 65℃. Afterwards, the membrane was washed in SSC/SDS according to the following scheme to remove the unspecific bound DNA: 1) 2 × SSC/0.1% SDS for 10 min at 20℃; 2) 2 × SSC/0.1% SDS for 30 min at 65℃; 3) 1 × SSC/0.1% SDS for 30 min at 65℃; and 4) 0.2 × SSC/0.1% SDS for 20 min at 65℃. To visualize the hybridized radioactive DNA, the membrane was wrapped in a household foil and then autoradiographed with a Kodak Xomat AR film and two amplified foils for 1-5 days at -70℃.

2.14.2. *in situ* Hybridization

2.14.2.1 cDNA Labeling with Digoxigenin (DIG)

For the DIG labeling of cDNA, the "Random Primer" DNA labeling method was again used. The reagents were purchased from Boehringer Mannheim. First, about 30 ng DNA, which had previously been purified and linearized, was in an Eppendorf tube filled with bidistilled water up to 15 μl, then denatured by heating in boiling water bath for 10-15 min and quickly cooling on ice/NaCl. It was followed by adding 2 μl hexanucleotide mixture, 2 μl dNTP labeling mixture (dATP, dCTP, dGTP each 1 mM and dTTP 0.65 mM as well as DIG-dUTP 0.35 mM), and then 1 μl Klenow enzyme (2 units/μl). The mixture was shortly centrifuged and incubated for 20 h at 37℃. After the incubation, 2 μl 0.2 M EDTA solution (pH 8.0) was given into it to stop the synthesis. The solution was mixed with 2.5 μ1 4 M LiCl and 75 μl pre-cooled (-20℃) absolute ethanol and left to stand 1 hour at -70℃ or overnight at -20℃ to precipitate DNA. It was followed by a 10 min centrifugation at 12000 *g*. The precipitation was washed one

time with 50 µl cold ethanol (70%), vacuum-dried and finally dissolved in 50 µl TE buffer. The amount of DIG-labeled DNA was determined in different dilutions (10×, 100×, 1000×, 2000×, 4000×, 8000×, 16000× and 32000×) in comparison to the standard DIG-DNA in dot-blot.

Hexanucleotide mixture:	Tris-HCl	0.5 M
	$MgCl_2$	0.1 M
	Dithioerythritol	1 mM
	BSA	2 mg/ml
	Hexanucleotide	62.5 A_{260} U/ml
	pH 7.2 (20°C)	

2.14.2.2 Cell Fixation and Permeabilization

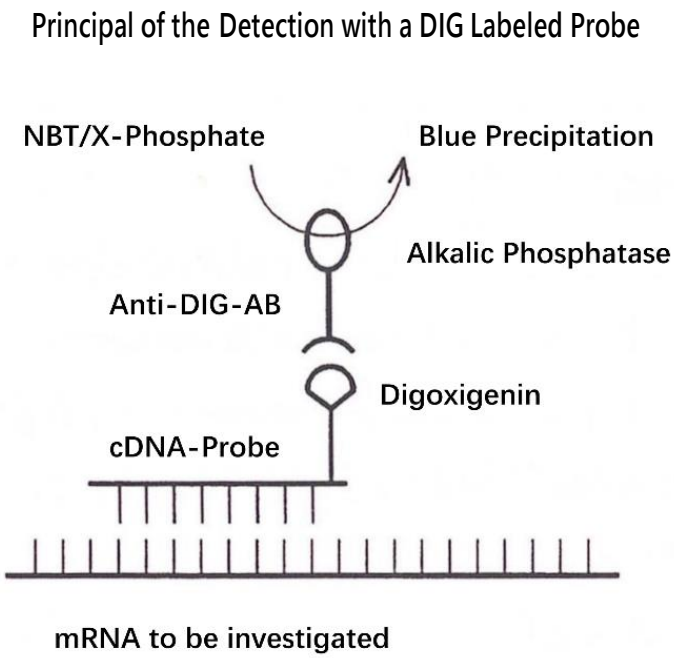
The frozen cytopreparations were immediately fixed for 10-15 min in 100% isopropanol. At room temperature, the fixed preparations were flushed first one time with PBS + 0.02% Triton X-100 (DEPC treated) for 15 min and then twice with 2 × SSC (DEPC treated) for 10 min.

2.14.2.3 Hybridization

The cytopreparations were pre-hybridized at RT with 25-35 µl hybridization buffer (2.8.3.6) for one hour. It was followed by 16-20 hours hybridization at 37°C with DIG-labeled cDNA probe (5 ng/preparation) that had been previously denatured at 100°C for 10 min and then quickly cooled down. At the end of the hybridization, the cytopreparations were washed as follow: first in PBS + 0.02% Triton X-100 at RT for 5 min; then in 2 × SSC at RT for 10 min; thereafter twice in 1 × SSC + 0.01% SDS at 50°C for 10 min, finally in 0.5 × SSC + 0.01% SDS at RT for 10 min.

2.14.2.4 Immunological Detection with anti-DIG Antibody

Right after the washing, the cytopreparations were incubated first with the blocking-solution (0.2% BSA + 0.3% Triton X-100 in TBS, pH 7.6) for 30 min at RT and then with anti-DIG antibody (1：1000 diluted in blocking-solution) one hour at 37℃. After the incubation, the cytopreparations were washed in TBS + 0.1% Triton X-100 for 15 min, and thereafter in TBS alone for 15 min, then equilibrated in TMS (pH 9.5, see 2.8.2.6) for 5 min. Subsequently, they were covered with NBT + X-Phosphate solution (4.5 µ1 Nitro blue tetrazolium salt, 75 mg/ml in 70% DMF + 3.5 µl 5-Brom-4-chlor-3-indoly phosphate p-toluidine-salt, 50 mg/ml in 100% DMF + 1 ml TMS buffer). The color reaction was carried out for 4-20 h at 37℃ in the dark. Then the cytopreparations were flushed with TE buffer (2.8.3.9) to stop the reaction, dehydrated in isopropanol, and purified with xylol for 40 seconds. The cytopreparations could be covered with glycerin-gelatin solution for microscopic examination.



2.15. Statistical Analysis

All experiments were done in triplicates and repeated at least three times. The proven differences between the samples were analyzed by means of “Student′s *t*-test”. $P < 0.05$ was considered as significant.

2. RESULTS

3.1. Enrichment and Culture for NK-Active Cells from Bone Marrow

3.1.1 Enrichment of NK Precursors from the Whole Bone Marrow Cells

As already mentioned in Introduction 1.1, the precursors of NK cells are found in the bone marrow. *In vitro* stimulation of freshly isolated bone marrow cells with IL-2 leads to the development of NK-inactive precursors to active NK cells. To detect the NK activity in mouse, the cytotoxicity against NK-sensitive Yac-1 tumor cells (see 2.9.6) is measured in a 4-hour test [Koo et al, 1986; Pollack & Rosse, 1987]. In the present study, the NK precursors, whose cell density is relatively low [Silvennoinen et al., 1986], were enriched by centrifugation in the density gradient. The freshly isolated whole bone marrow cells were separated into 4 fractions via a 4-layer Percoll gradient (Gradient I, see 2.10.2). Then all fractions were cultured with IL-2. After 48- as well as 72-hour incubation, the cytotoxicity of the cells against Yac-1 was tested. So far, the highest anti-Yac-1 activity (**Tab. l**) was found in the cells in the light Percoll fraction (Fr. 32%/52%). The cytotoxic activity was related to the presence of IL-2 and did not appear in the culture without IL-2.

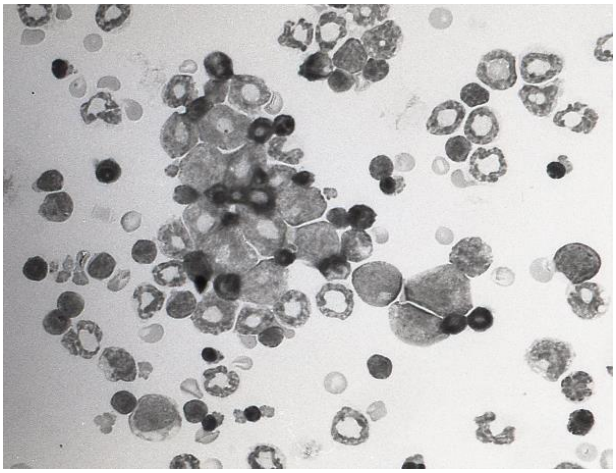


Fig. 1. Freshly isolated whole bone marrow cells. The cytopreparation shows a heterogeneous population that consists of different cell types (myeloid monoblasts and poly-morphonuclear cells, erythrocytes, small lymphocytes, and as yet unidentifiable progenitor cells).

Tab. l. ***Cytotoxicity of Different Percoll fractions from Murine Bone Marrow against Yac-1 Tumor Cells after IL-2 Stimulation***

Cells	in Complete Medium		in 100 U/ml IL-2	
	48 h	**72h**	**48 h**	**72 h**
whole BM cells	0%	0%	2±2%	15±3%
Fr.1 32%/52%	0%	0%	15±2% [a]	35±5% [b]
Fr. 2 52%/55%	0%	0%	2.5±2%	5±3%
Fr. 3 55%/60.6%	0%	0%	0%	0%
Fr. 4 60.6%/bottom	0%	0%	0%	0%

The cells were plated in 96-well microtiter plates (2.5×10^5 cells/well) and incubated either with complete medium alone or with complete medium + 100 U/ml IL-2. After appropriate incubation time, the cells were washed three times and further incubated with ^{51}Cr labeled Yac-1 (5×10^3 cells/well) for 4 hours. The results were expressed as % specific lysis of Yac-1 target cells (see 2.12.2).

[a] $p < 0.01$, Fr. 32%/52% vs. Fr. 52%/55%

[b] $p < 0.01$, Fr. 32%/52% vs. Fr. 52%/55%

3.1.2 Microscopic Investigation of Bone Marrow Cells in Different Percoll fractions

The freshly isolated whole BM cell population consists of heterogeneous cell types (see **Fig. 1**). It was separated by centrifugation over a Percoll gradient into four fractions, each of which contained cells of similar density. Cytopreparations (2.11.1) of all Percoll

fractions were made, stained according to Pappenheim Staining (2.11.6) and finally the cell composition of each fraction was examined microscopically. The results are demonstrated in **Table 2**. A large proportion of the cells in the light fraction (32%/52%) could not be identified due to their immature morphology[①]. Such unidentifiable precursors were hardly found in the heavy Percoll fractions (52%/55%[②], 55%/60% and 60%/bottom).

Tab. 2. ***Cell Types in Different Fractions of Percoll Gradient I***

Percoll Fr.	**Unidentifiable Precursors**	**Monoblast-like Cells**	**Polymorphnuclear Cells**	**Small Lymphocytes**	**Erythrocytes**
32%/52%	60±10% *	15±5%	10±5%	5±3%	2±1%
52%/55%	10±5%	9±5%	39±12%	25±10%	8±5%
55%/60.6%	–	1±1%	60±10%	10±5%	30±10%
60.6%/bottom	–	–	–	15±3%	85±5%

"–" means the equivalent cell type was not observed in the fraction.

* $p < 0.02$ Fr. 32%/52% vs. Fr. 52%/55%

3.1.3 Proliferation of the Cell Population Containing NK Precursors

Cultivation of bone marrow cells from the light Percoll fraction (32%/52%), which contained the most NK precursors, with lymphoid growth factor IL-2 did not lead to significant proliferation. In contrast to it, the cultivation of this cell fraction with CSF-

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①See Additional Figure 1 on page 126 (not shown in the original dissertation)

②See Additional Figure 2 on page 126 (not shown in the original dissertation).

containing medium (L cell-conditioned medium) resulted in a strong proliferation response (**Fig. 2**). Since L cell-conditioned medium could contain factors other than CSF (CSF-1 and GM-CSF), the recombinant colony-stimulating factors, namely human recombinant (hr) CSF-1 and murine recombinant (mr) GM-CSF, were used for further proliferation test. **Figure 3** shows that both hr CSF-1 and mr GM-CSF stimulated strong cell proliferation. These results indicate that the cells in the light Percoll fraction grow dependent on myeloid growth factor.

Proliferation of BM Cells of Percoll fraction 32%/52%

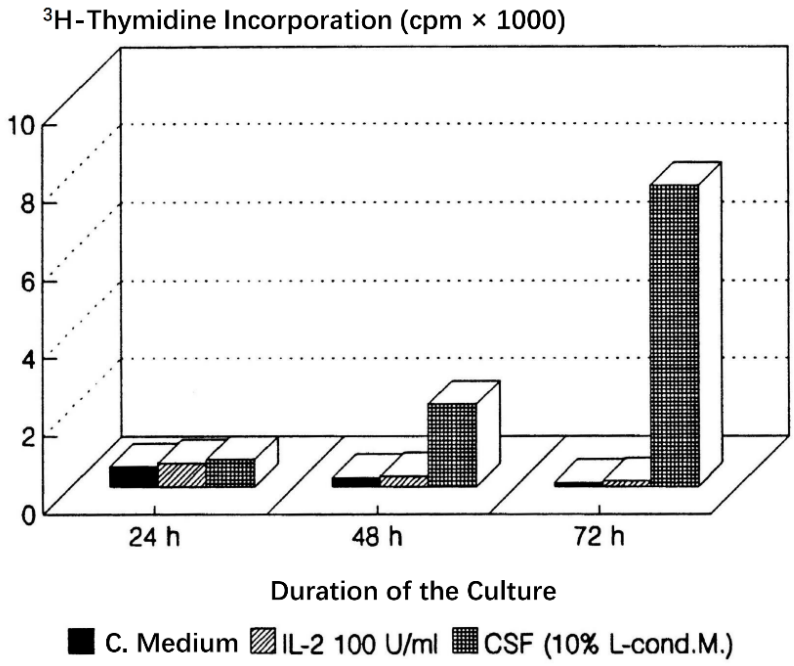


Fig. 2. The cells were plated in 96-well microtiter plates at 5×10^3 cells/well and incubated with 1) complete medium (C. Medium) alone; 2) IL-2 (100 U/ml in complete medium); or 3) CSF-containing L cell-conditioned medium (L-cond. M.). The [^{3}H]-Thymidine incorporation assay (2.12.1) was performed after the incubation time indicated.

Cell Proliferation in the Presence of Recombinant CSFs

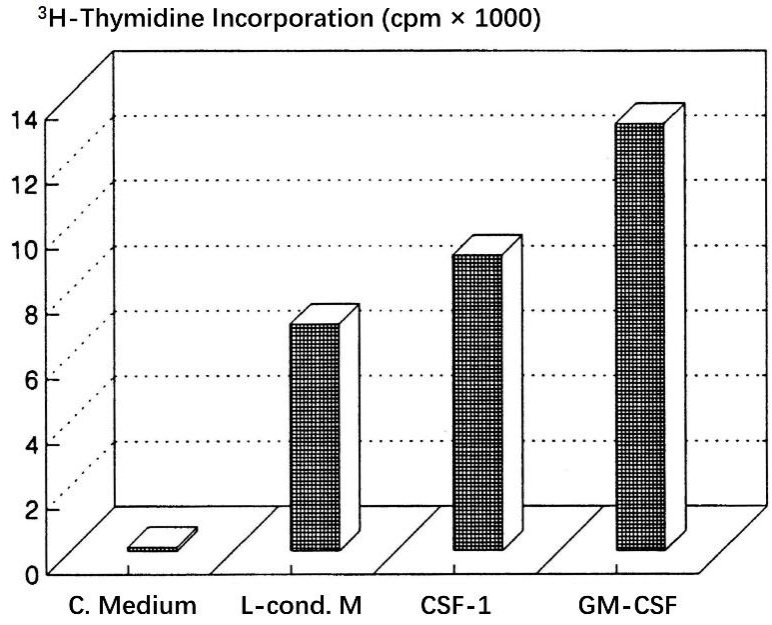


Fig. 3. The cells of Fr. 32%/52% were incubated in 96-well microtiter plates (5×10^3 cells/well) with, in the order described in the figure, 1) complete medium（C. Medium）； 2) 10% L cell-conditioned medium (L-cond. M.); 3) 1000 U/ml hr CSF-1； und 4) 1000 U/ml mr GM-CSF. Complete medium and L cell-conditioned medium served as controls. After 2 days, [^{3}H]-Thymidine was added into the culture and 20 h later the incorporation rate was measured.

3.1.4 Effective Bone Marrow Culture for Proliferating and NK-Active Cells

Under the culture condition only with CSF, none cytotoxic activity of the cells (Fr. 32%/52%) was observed. After a 3-day culture, the cells were separated into five fractions by Percoll gradient II (2.10.2), and the lytic activity of each fraction was tested. No Yac-1 lysis could be detected. Since the cells showed good NK activity after culturing in the presence of IL-2 as reported under 3.1.1, an attempt was made to use a

combination of CSF (as cell growth factor) with IL-2 (as NK activity stimulating factor) in the culture. This culture condition with two factors led to both active cell proliferation and NK activity. **Figure 4** demonstrates that the efficacy of IL-2 was strongly influenced by the time of addition. The earlier IL-2 was added into the culture, the more effective induction of NK activity was observed. When IL-2 was added only two days after the start of the culture, NK activity could no longer be induced in the cells.

Influence of the Timing of Addition of IL-2
on the Induction of NK activity

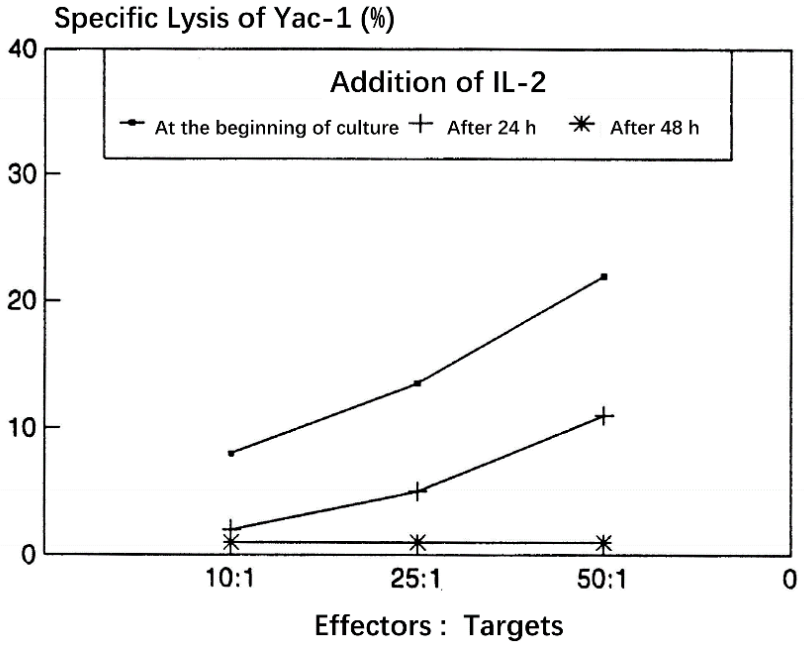


Fig. 4. The cells of Fr. 32%/52% were cultivated in three separate cultures with CSF (10% L cell-conditioned medium). IL-2 (100 U/ml) was added respectively into the relevant cultures at three different time points: 1) Right at the beginning of the culture; 2) 24 hours later; and 3) 48 hours later. Each culture was incubated with IL-2 for 72 hours. Then a 4-hour cytotoxicity test (see 2.12.2) was performed.

3.1.5 Purification of NK-Active Cells after Culture in CSF + IL-2

After a 3-day BM pre-culture in the "cocktail" of CSF with IL-2 (see 2.10.3), the cells were harvested from the culture and fractionated via a 6-layer Percoll gradient II (2.10.2). The resulting five cell fractions were used for the morphological identification and the cytotoxicity test. It was proved that the NK-active cells were again enriched in the light fractions (Fr. 1-3, see **Tab. 4**). Thereby the fraction 2 (40.8/45.3%), which clearly contained the highest percentage of macrophage precursors (**Tab. 3**), exhibited the best NK Activity (**Tab. 4**). In this fraction, about 60% of the cells harvested from the CSF + IL-2 pre-culture were regained (**Tab. 4**).

Tab. 3. ***Cell Composition in the Fractions of Percoll Gradient II***

Percoll Fr.	Macrophage-Precursors*	Slight ad-herent Mϕ	PMN	lymphocytes + others
1. 32.0/40.8%	20±7%	67±9%	5±2%	3±3%
2. 40.8/45.3%	80±5% [a b]	8±4%	7±3%	5±3%
3. 45.3/50.5%	42±5%	3±1%	40±6%	10±2%
4. 50.5/55.0%			80±4%	20±8%
5. 55.0/60.6%			48±7%	44±11%

* The morphological characteristics of macrophage precursors have been described in previous work [Lohmann-Matthes 1979; Baccarini et al. 1985].

[a] $p < 0.01$ Fr.2 vs. Fr. 1

[b] $p < 0.01$ Fr.2 vs. Fr.3

Tab. 4. ***NK Activity*** **and** ***Cell Number in Each Fraction of Percoll Gradient II***

	Fr. 1	**Fr. 2**	**Fr. 3**	**Fr. 4 + Fr. 5**
NK Activity（$LU/10^7$）*	15±4	33±4 [a b]	20±3	0
Cell number/whole	3±1%	60±7%	27±2%	7±3%

* The lytic units in the cell populations ($LU/10^7$) were calculated as described under 2.12.2.

[a] $p < 0.05$ Fr. 2 vs. Fr. 1

[b] $p < 0.05$ Fr. 2 vs. Fr. 3

Since the Fraction 2 had both the best NK activity and the highest purity (**Tab. 3**), it was used for further investigations. To increase the purity of this fraction, namely to remove the PMN, slight adherent macrophages and small lymphocytes, the cell population was first incubated with iron particles (2.10.5) in a plastic culture dish for 30 min at 37°C to separate the adherent and phagocytic cells (PMN and macrophages) from the population. Then, as described under 2.10.6, an antibody "cocktail" of rat anti-mouse B220 and rat anti-mouse CD3 was incubated with the cells for 45 min on ice. After the incubation, the cells that had bound B220 or CD3 antibody (typical B and T lymphocytes) were eliminated from the population with Dynal Beads coupled with sheep anti-rat IgG. Microscopic examination of the cells treated in this way revealed that they are homogeneous macrophage precursors (**Fig. 5**). No loss of NK activity was observed after this purification process. The purified MP cells differentiated into mature macrophages (**Fig. 6**) after a 24-hour incubation at 37°C with 0.2 µg/ml PMA, which can quickly cause the end differentiation of macrophage lines [Nguyen et al. 1993].

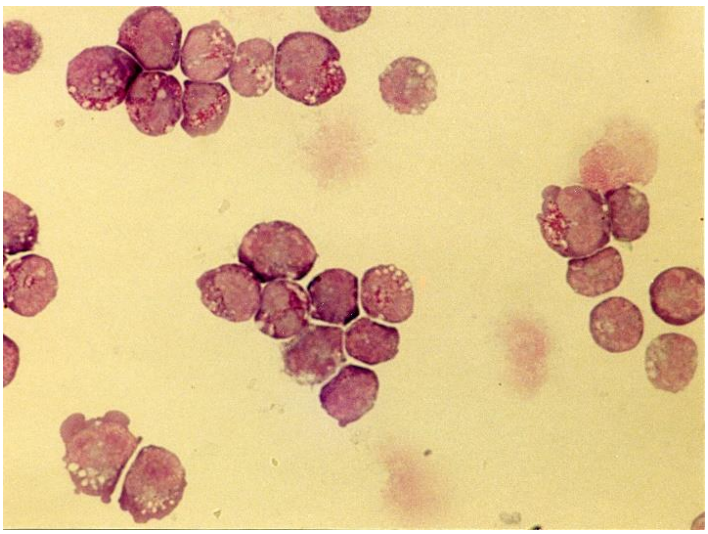


Fig. 5. Morphology of purified macrophage precursors (MP cells). The cells were isolated from a 3-day BM pre-culture with CSF + IL-2 (200 U/ml GM-CSF + 100 U/ml IL-2, translation notes) and purified as described under 2.10.4-6.

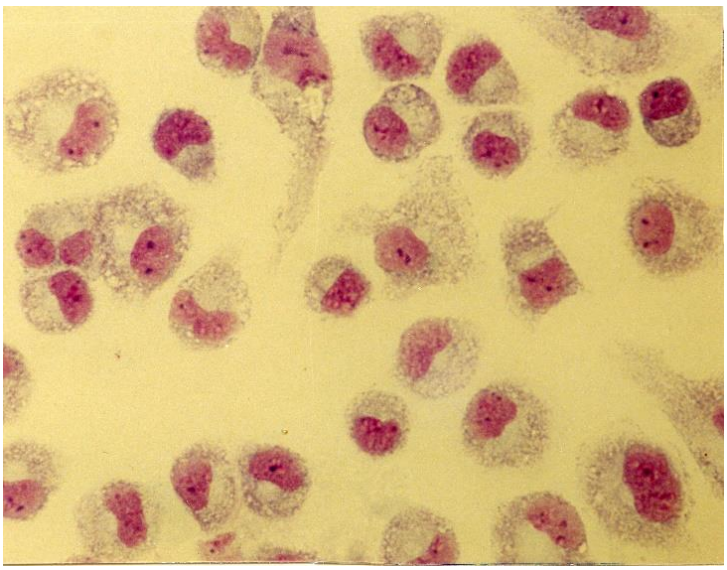


Fig. 6. The macrophages matured from the purified MP cells. The MP cells, isolated and purified from 3-day BM pre-culture with CSF + IL-2 (200 U/ml GM-CSF + 100 U/ml IL-2, translation notes), were incubated with PMA (0.2 µg/ml) in 8-well chamber-slides (see 2.11.2) at 37°C in 5% CO_2 for 24 h.

2.2.Properties of IL-2 Induced, NK-Active Macrophage Precursor Cells

NK-active MP cells, isolated from 3-day BM pre-culture with CSF + IL-2, were further characterized. In the following, the cultures that started with the MP cells isolated from 3-day BM pre-culture as starting population were called "main-culture".

3.2.1 Morphology and Phenotype of NK-Active Macrophage Precursor Cells

When looking at the cells in a light microscope with high magnification (> 600×), it turned out that the NK-active MP cells contain cytoplasmatic granules (**Fig. 7**), which are not found in NK-inactive macrophage precursors, isolated from BM pre-culture with CSF alone (**Fig. 8**).

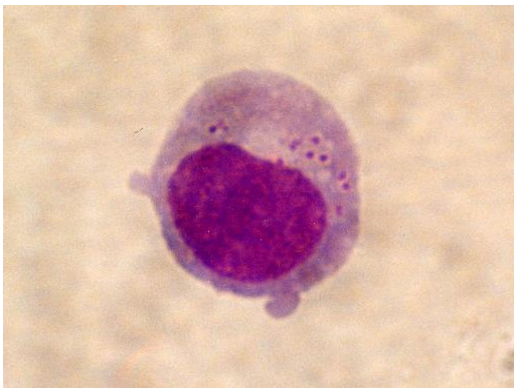


Fig. 7. The cytoplasmatic granule-containing, NK-active MP cell obtained from the BM pre-culture with CSF + IL-2.

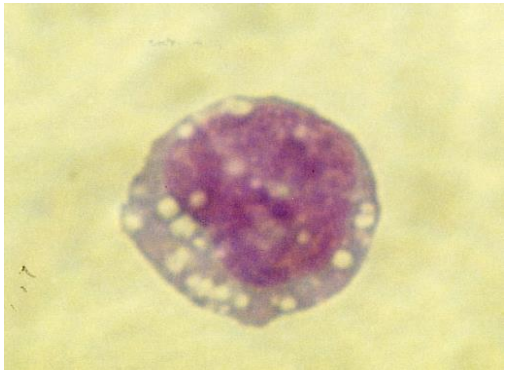


Fig. 8. The agranular, NK-inactive MP cell obtained from the BM pre-culture with CSF alone.

To investigate the phenotype (surface antigen profile) of purified NK-active MP cell population and at the same time check their purity, the cells were stained with various specific monoclonal antibodies using a direct or indirect fluorescence staining method (2.11.4). The selected antibodies were following: 1) NK-1.1, anti-mouse NK cells; 2) F4/80, anti-mouse macrophages; 3) anti-mouse Mac-1, anti-myeloid cells; 4) FITC-anti-mouse Lyt-1, anti-all T cells; 5) FITC-anti-mouse Lyt-2, anti-cytotoxic T cells; 6) FITC-anti-mouse L3T4, anti-T helper cells; 7) anti-mouse B220, anti-B cells; and 8) Mouse IgG 2b anti-human monocytes Leu-M3, as isotype control. The results of the fluorescence staining were analyzed and evaluated with the help of FACS. In **Figure 9**, it is demonstrated that the purified NK-active MP cell population was completely negative for Lyt-1, Lyt-2, L3T4, and B220. The cells had NK-1.1 and Mac-1 markers on their surface, but showed only a weak expression of F4/80. F4/80 is a marker of macrophage differentiation and only to be found clearly on mature macrophages [Crocker & Gordon 1985; Hoefsmit et al., 1986].

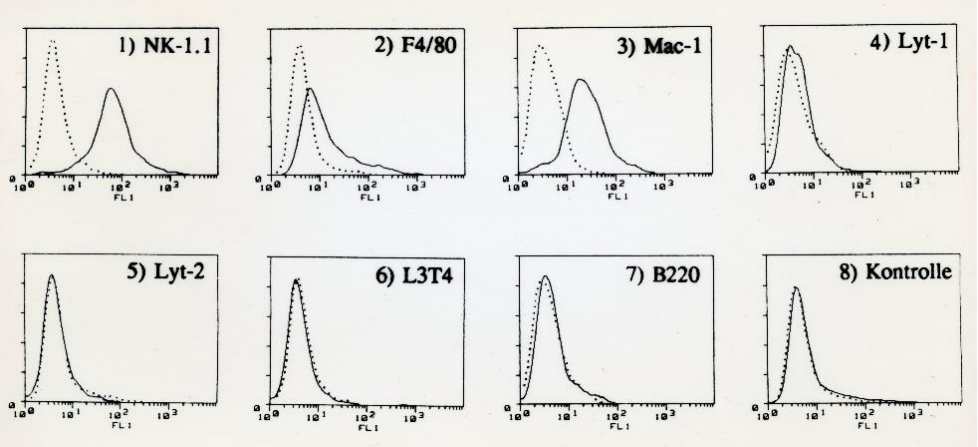


Fig. 9. Surface antigen profile of NK-active MP cell population. The purified NK-active MP cells from BM pre-culture with CSF + IL-2 (200 U/ml GM-CSF + 100 U/ml IL-2, translation notes) were examined by means of the Immunofluorescence staining. A mouse IgG 2b served as isotype control. All cells in the population were analyzed.

3.2.2 Factor Dependence of Cell Proliferation

Since the NK-active MP cells showed similarities (LGL morphology and NK-1.1^{+}) with the NK cells, a proliferation test was carried out to determine which cell stem-specific growth factor (CSF-1 or IL-2) is needed for further proliferation of the cells. In this test, MP cells from 3-day BM pre-culture (with CSF + IL-2) were seeded in 96-well microtiter plates, then incubated either with 1000 U/ml IL-2 or with 1000 U/ml CSF-1 or with IL-2 + CSF-1 (1000 U/ml each). 1000 U/ml IL-2 was used in this experiment because it was described that NK cells require high concentration of IL-2 for proliferation [Talmadge et al. 1986]. On day 1, 2, 3, 4 of the culture, the [^{3}H]-Thymidine incorporation assay was performed. **Figure 10** indicates that the NK-active MP cells still relied on CSF-1 for their proliferation. IL-2 had neither stimulation nor inhibition effect on the cell proliferation during the tested period (from day 1 to 4).

Factor Dependence of Proliferation of NK-like MP Cells

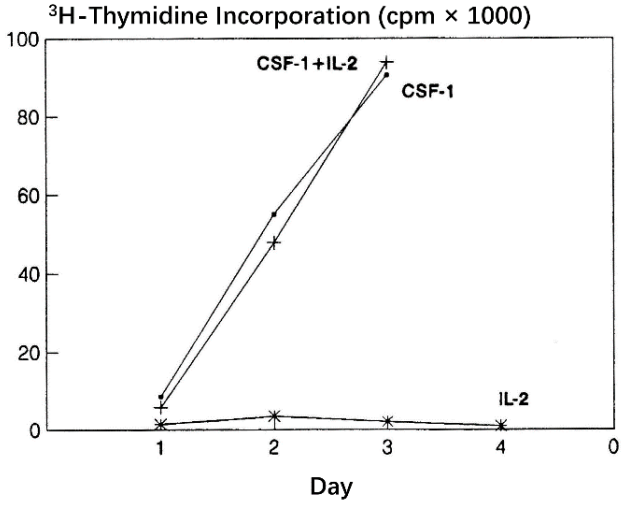


Fig. 10. 5 × 10^{3} cells in 200 µl complete medium per well were seeded in 96-well microtiter plates and incubated with: 1) IL-2 (1000 U/ml); 2) CSF-1 (1000 U/ml); and 3) IL-2 + CSF-1 (1000 U/ml each). The [^{3}H]-Thymidine incorporation was tested on different days.

3.2.3 Factor Dependence of Cytotoxicity

In order to study the culture conditions under which the NK-active MP cells keep their cytotoxicity and how they may change, the following investigations were carried out.

In the first investigation, the NK-active MP cells were seeded in microtiter plates (1×10^5 cells/well) and cultivated in the presence of either 100 U/ml IL-2 or 100 U/ml CSF-1, or IL-2 + CSF-1 (100 U/ml each). These microcultures were feed every other 2 days with fresh complete medium and the relevant factors. The cytotoxicity of the cells against Yac-1 was tested on day 2, 4, and 6 of the culture. **Figure 11** demonstrates that the NK-active MP cells in the culture with CSF-1 alone quickly lost their cytotoxicity. In the culture with IL-2 alone, NK activity stayed well. The combination of CSF-1 + IL-2 led to an increased cytotoxicity. Since CSF-1 did not have a stimulating effect on the NK activity, the increasing of cytotoxicity in the culture with CSF-l + IL-2 is presumably caused by the increase of the number of effector cells.

Kinetics of NK Activity in the Presence of CSF-1 + IL-2

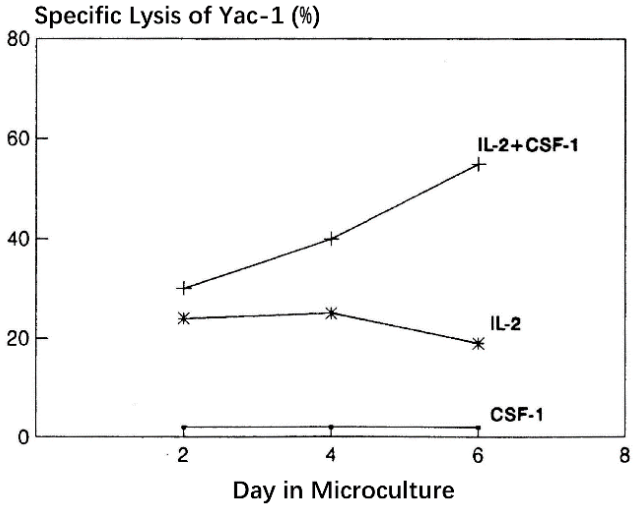


Fig. 11. In 96-well microtiter plates, NK-active MP cells were cultivated respectively with 100 U/ml IL-2, 100 U/ml CSF-1, and IL-2 + CSF-1 (100 U/ml each). NK activity against Yac-1 was tested on different days.

A high NK activity could be achieved after 5 days of main-culture with CSF-1 + IL-2 (100 U/ml each). At this time, the lytic units in the cell population are increased several times in comparison to the NK-active MP starting population that was freshly isolated from the BM pre-culture. For the second investigation on the factor dependence of cytotoxicity, these strong lytic effector cells were transferred into microcultures and incubated with or without IL-2 in CSF-1 or complete medium overnight. Then the cytotoxicity against Yac-1 was tested. As shown in **Figure 12**, the cytotoxicity was found again only in the cultures to which IL-2 had been given. The highly NK-active cells lost their cytotoxicity when incubated overnight without IL-2. It is excluded that the effector cells had died during withdrawal of IL-2, because no cell death was observed on light microscopic examination. These results indicate that the cells can keep their NK activity only in the presence of IL-2.

Loss of Cytotoxicity after Withdraw of IL-2

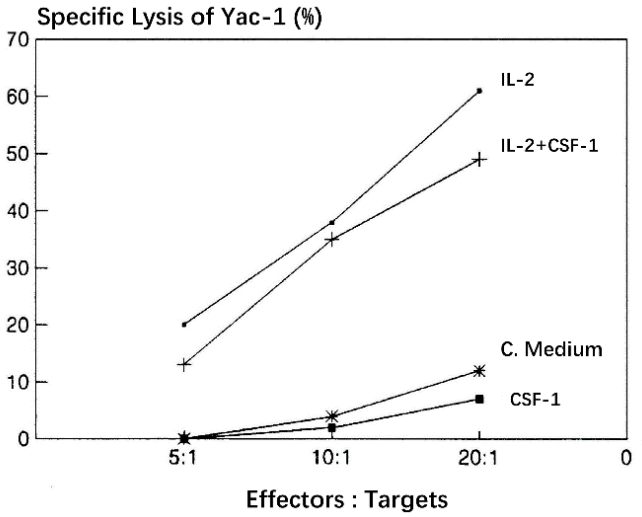


Fig. 12. Highly NK-active cells from 5-day main-culture with CSF-1 + IL-2 were transferred into 96-well microtiter plates and incubated overnight with: 1) CSF-1 (100 U/ml); 2) complete medium; 3) IL-2 (100 U/ml); and 4) IL-2 + CSF-1 (100 U/ml each). The cytotoxicity test against Yac-1 was performed the next day.

3.2.4 Target Cell Selectivity of Effector Cells

To investigate whether the NK-like MP cells exert their lytic activity only selectively on NK-sensitive target cells (represented by Yac-1), they were used as effectors in a cytotoxicity test against P815 mastocytoma (NK-resistant tumor line). The incubation period of effector cells with the tumor cells was prolonged from 4 hours to 16 hours, because this is the optimal lysis time for P815 cells. Yac-1 cells served thereby as positive control. As shown in **Figure 13**, the effector cells, while highly lytic against Yac-1 target cells, were not able to kill P815 tumor cells.

Target Selectivity of NK-Active MP Cells

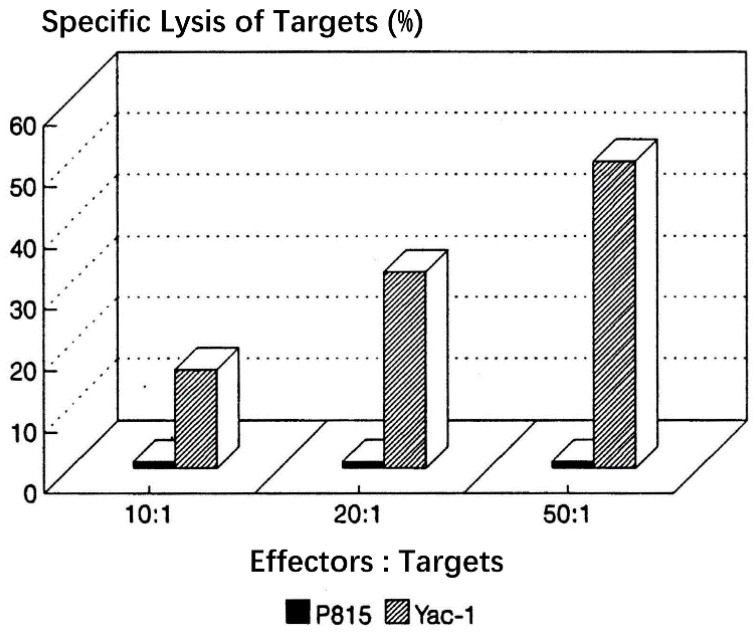


Fig. 13. For the test, the effector cells were incubated in 96-well microtiter plates respectively with ^{51}Cr-labeled P815 and Yac-1 tumor cells at 37°C in 5% CO_2 for 16 h.

3.2.5 Morphological Development of Cells during Withdraw of IL-2

The chapter on factor dependence of cytotoxicity (3.2.3) showed that, in the cultures with different lymphokine supplements, the same MP starting population developed into functionally different behaving cells (see **Fig. 11** and **12**). To find out whether the cells from the cultures with CSF-1 alone or with CSF-1 + IL-2 are morphologically different, the MP cells from 3-day CSF + IL-2 BM pre-culture (200 U/ml GM-CSF + 100 U/ml IL-2, translation notes) were seeded in "chamber-slides" (see 2.11.2) and further cultivated either with CSF-1 + IL-2 (200 U/ml CSF-1 + 500 U/ml IL-2, translation notes) or with CSF-1 (200 U/ml, translation notes) alone. The cultures were feed two days later and finished on day 4. After a short centrifugation at 1000 rpm, the supernatants in “chamber” were carefully removed and the slides were air dried. Subsequently, they were stained according to Pappenheim staining (2.11.6) and examined by light microscope. **Figure 14** and **16** show that the cells from both cultures look totally different. In the culture with CSF-1 alone, the NK-like MP cells differentiated into the cells that exhibit typical morphology (**Fig. 14**) and phenotype (**Fig. 15**) of macrophages. In contrast, the same precursor cells in the culture with CSF-1 + IL-2 developed into cells with numerous cytoplasmatic granules (**Fig. 16**).

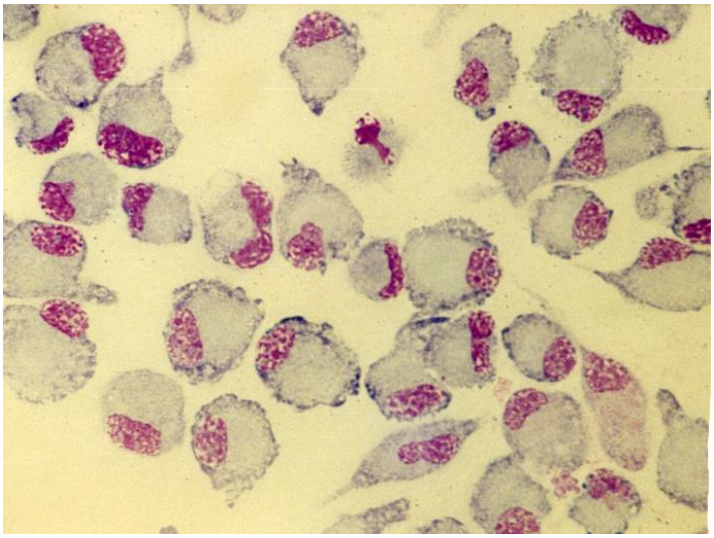


Fig. 14. The macrophages matured from NK-like macrophage precursors after 3-day culture with only CSF-1（200U/ml, translation notes）.

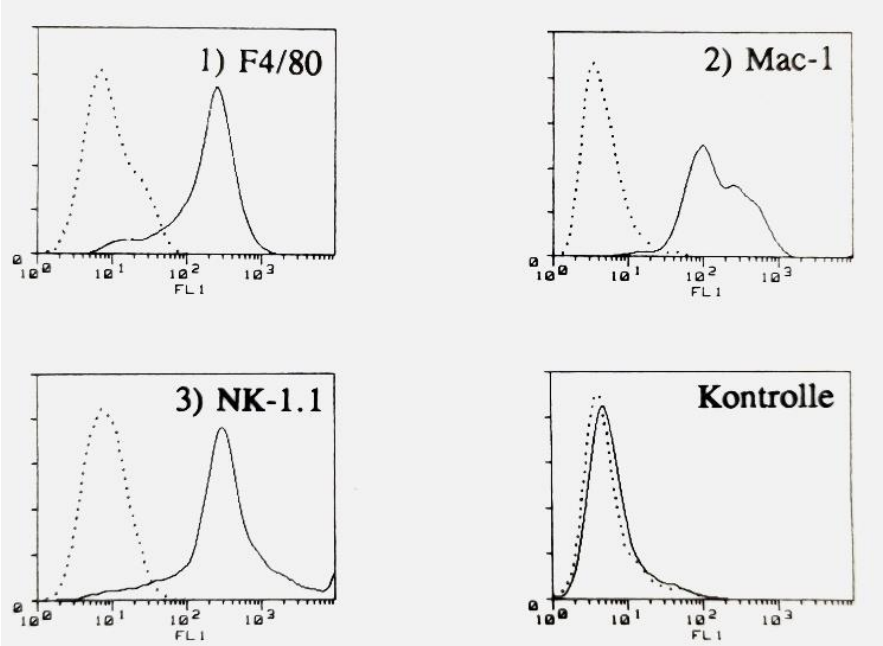


Fig. 15. Phenotype (surface antigen profile) of mature macrophages obtained from NK-like macrophage precursors.

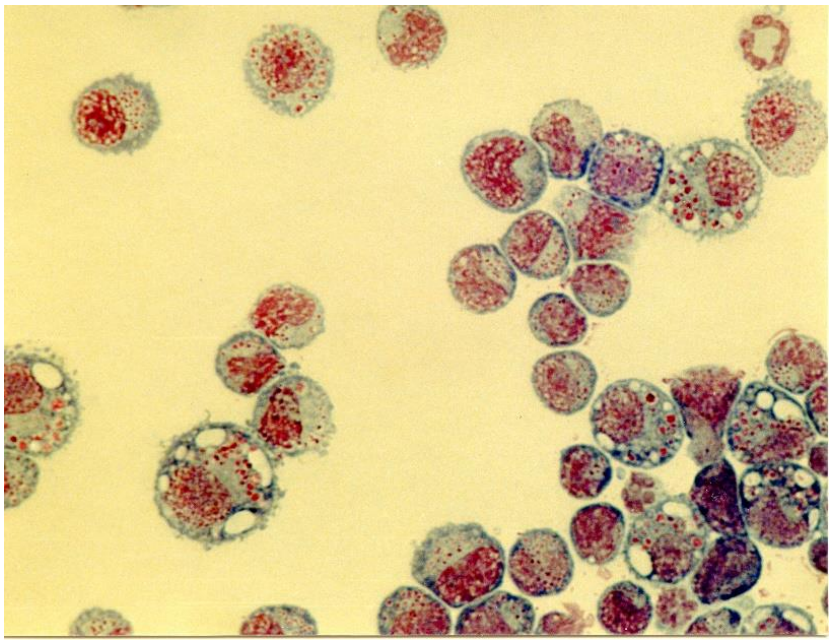


Fig. 16. Morphology of the cells developed from NK-like macrophage precursors after 3-day culture with CSF-l + IL-2 (200 U/ml CSF-1 + 500 U/ml IL-2, translation notes).

3.3. Induction of LAK Activity in NK-Like Macrophage Precursor Cells

The results presented in the previous chapters (3.2.1-5) have shown that the NK-active MP cells induced by IL-2 were functionally, morphologically and phenotypically similar to NK cells. In the following experiments, it was investigated how they developed further under the condition for the induction of LAK cells (see 1.3).

3.3.1 Activation of LAK Activity by Stimulation with High Dose IL-2

NK-like MP cells from BM pre-culture with CSF + IL-2 were cultivated in 9-cm culture dishes (Greiner) in CSF-containing medium (100 U/ml CSF-1) with high concentration of IL-2 (1000 U/ml). After 3-day LAK culture (see 2.10.7), the cells were collected, washed and for testing LAK activity plated in 96-well microtiter plates with different cell numbers of 1×10^5, 5×10^4, 2×10^4, and 1×10^4 per well. Both NK-sensitive Yac-1 and NK-resistant P815 tumor cells were suspended as ^{51}Cr-labeled target cells at a concentration of 1×10^5 cells/ml in complete medium and then pipetted 100 µl per well into the microtiter plates. This resulted in a series of E：T ratios of 10：1; 5：1; 2：1 and 1：1. The plates were shortly centrifuged at 600 rpm and in the end, due to the optimal lysis time for P815, incubated at 37°C in 5% CO_2 for 16 hours. For comparison, the same NK-like MP starting population was cultivated in medium with low concentration of IL-2 (100 U/ml) and tested in parallel. **Table 5** demonstrates that the cells incubated with 1000 U/ml IL-2 for 3 days were activated to lyse NK-sensitive and NK-resistant tumor cells. However, in the culture with 100 U/ml IL-2 only the NK-sensitive cells were lysed. This result indicates that by the stimulation with high dose IL-2 the NK-like MP cells developed into effector cells with LAK activity.

Tab. 5. ***Effect of IL-2 Doses on NK-Like MP Cells***

Culture condition	Cytotoxicity (LU/10^7 cells)	
	Yac-1	**P815**
100 U/ml CSF-1 + 100 U/ml IL-2	325	0
100 U/ml CSF-1 + 1000 U/ml IL-2	824	455

NK-like MP cells were cultivated under the presented culture conditions for 3 days, and then tested for the lytic activity against Yac-1 and P815 by a 16-hour cytotoxicity test. The lytic units were calculated as described under 2.12.2.

3.3.2 Kinetics of LAK Induction

NK-like MP cells were cultivated in 96-well microtiter plates (2×10^5 cells in 200 µl medium per well) with 1000 U/ml IL-2. These microcultures were feed every other 2 days with fresh medium and IL-2, and their LAK activity was tested on day 0, 3, 6, 9 and 12 of the LAK culture in order to follow the kinetics of LAK induction in the cells. **Figure 17** shows that the LAK activity in the culture increases in the first 3-6 days and then decreases despite continuous supplement of fresh medium and the stimulating factor IL-2. This result is consistent with the findings described by Grimm et al. that *in vitro* induced LAK activity by IL-2 is first detectable after 3 days of the culture and is only to be found in the culture for about one week [Grimm et al., 1982].

Kinetics of LAK Induction in IL-2 Stimulated MP Cells

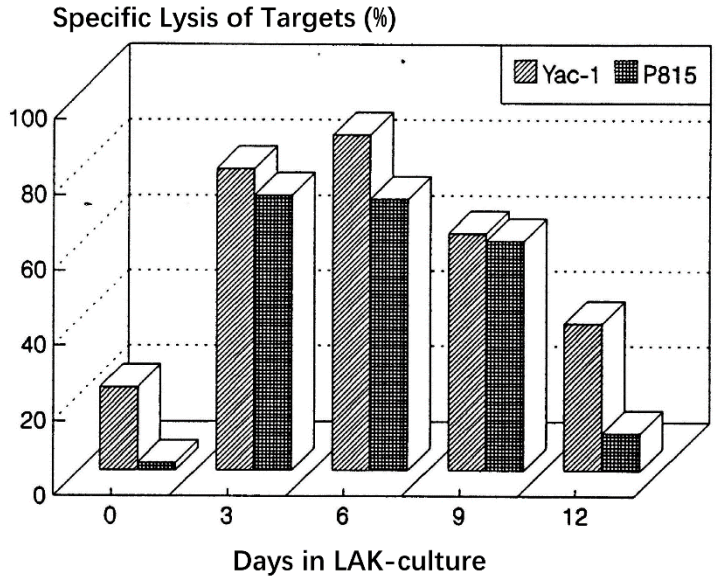


Fig. 17. 2×10^5 NK-like MP cells per well were incubated in 96-well microtiter plates with 1000 U/ml IL-2 (LAK-culture). On different days, ^{51}Cr-labeled target cells were given into the wells (1×10^4/well) and the LAK-activity of the cultures was examined by a 16-hour cytotoxicity test.

3.3.3 Morphology of LAK Cells Developed from NK-Like Macrophage Precursors

At the time points for LAK activity test (day 3, 6, 9 and 12), the cell samples were taken out of the LAK culture, spun on microscopic slides with the help of Cytospin centrifuge, air dried and stained according to Pappenheim staining. Microscopic investigation showed a remarkable morphological change of the cells: in these cells, a strong amplification of cytoplasmatic granules was observed (**Fig. 18**). The longer the LAK culture lasted, the denser and larger the granules were. Finally (approximately on day 8-10 of the culture) the LAK effectors that contained rich granules began to fragment

(**Fig. 19**). The decrease of LAK activity observed after 9 days of LAK culture (see **Fig. 17**) is due to the mass cell death in the culture.

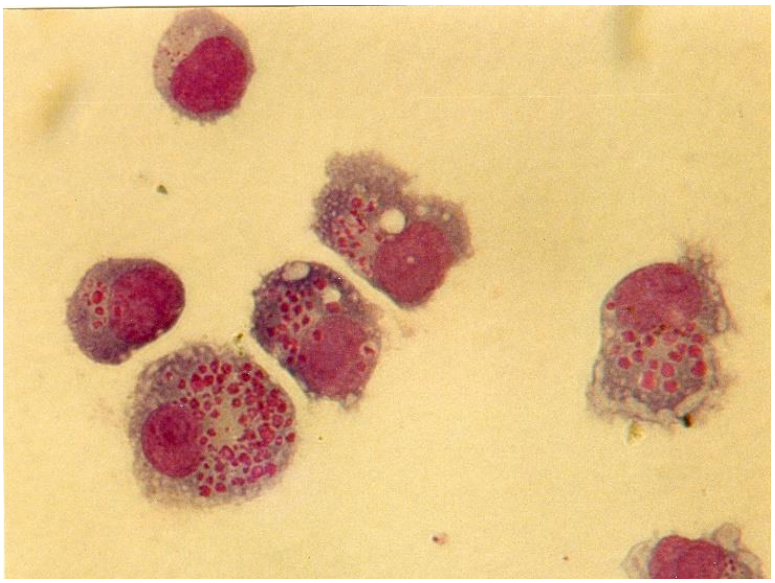


Fig. 18. Morphology of LAK effector cells isolated on day 4 of LAK culture.

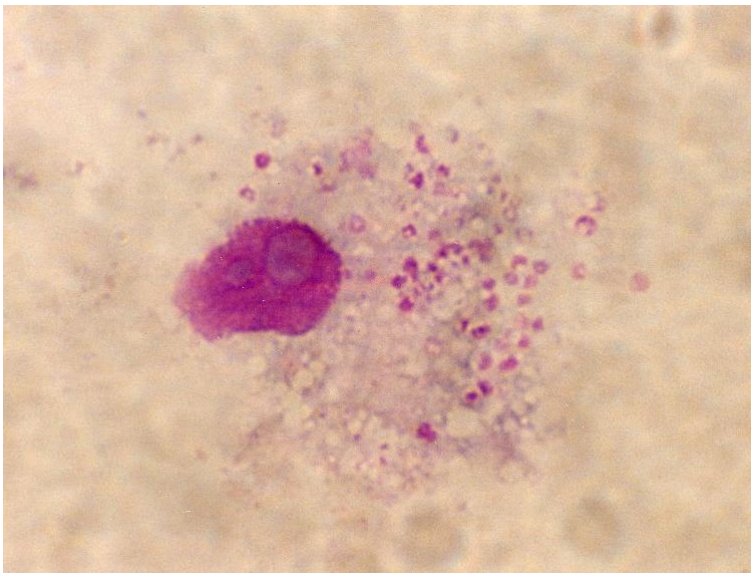


Fig. 19. Fragmentation of LAK cells observed on day 9 of LAK culture.

3.3.4 Phenotype of LAK Cells Developed from NK-Like Macrophage Precursors

The LAK cells to be investigated were harvested on day 4 of LAK culture that had started with the purified NK-like MP cells. At this time, the cells exerted high LAK activity and they were still in good condition. After three time washing, each of 1-5 × 10^5 cells were transferred into small polyethylene tubes (0.6 ml Greiner), and direct or indirect fluorescence staining with antibodies anti surface markers was performed. NK-1.1, F4/80, Mac-1, anti-CD3 and anti-CD8 were applied respectively for the staining to investigate the phenotype of the LAK effectors. At the same time, the possibility that lymphocytes might grow up in LAK culture could also be examined.

Figure 20 shows clearly that the LAK effectors, in comparison to the NK-like MP starting population (see **Fig. 9**), the Mac-1 and F4/80 markers had completely lost. These cells were positive for NK-1.1 but negative for T cell markers CD3 and CD8, a typical phenotype of the described LAK cells induced from NK cells [Hercend & Schmidt, 1988].

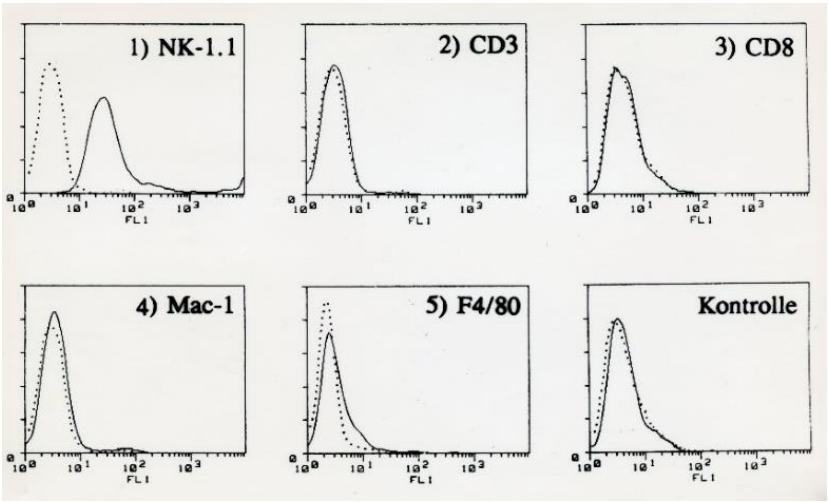


Fig. 20. Phenotype (surface antigen profile) of LAK effector cells isolated on day 4 of LAK culture.

3.4. Perforin Production by Macrophage Precursor Cells

In the previous experiments, it could be shown that the appearance of cytoplasmatic granules was closely related with the cytotoxic function of the cells. Now it should be proved whether these granules contain the lytic protein perforin. For this purpose, the following studies were carried out.

3.4.1 Calcium-Dependence of Cytotoxicity

A key feature of perforin-mediated cytotoxicity is its calcium-dependence: in the absence of calcium, the cytotoxicity does not appear. It has been described that perforin is structurally and functionally similar to the complement component C9 [Tschopp & Nabholz 1990]. In the presence of calcium, perforins make holes in the cell membrane of target cells and lyse the target cells. Therefore, a calcium-dependence of cytotoxicity is considered as an indication of the effect of perforin.

As described in materials and methods (2.12.3), three test media were prepared: depleted medium without free Ca^{2+} ions by adding 10 mM EDTA, reconstituted medium by adding $CaCl_2$ (15 mM final concentration) to the depleted medium, and complete medium. In these three media, the cytotoxicity test against Yac-1 cells was respectively performed using the effector cells from 4-day main-culture with CSF-1 + IL-2 (500 U/ml each). The results are demonstrated in **Figure 21**, it is clear that the presence of calcium is essential for the lysis of target cells.

Calcium-Dependence of Cytotoxicity of NK/LAK-Like MP Cells

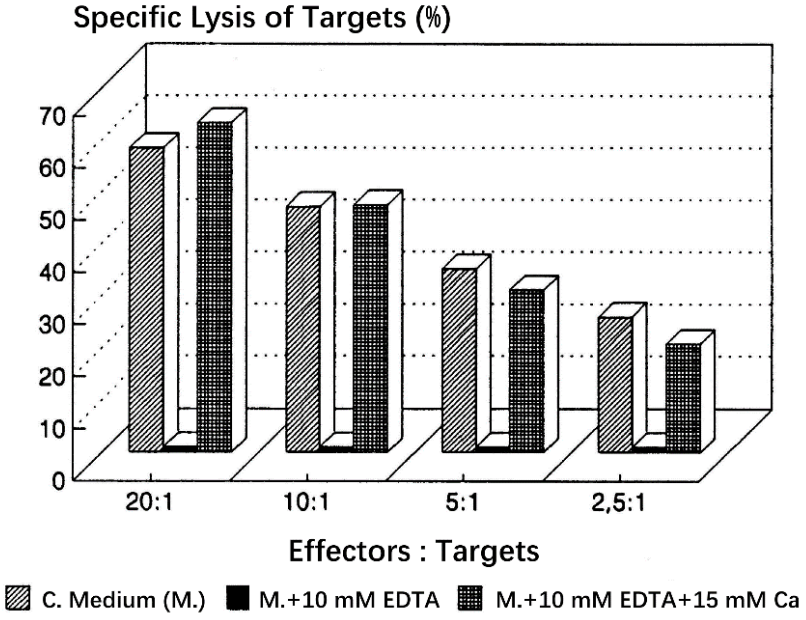


Fig. 21. The effector cells were harvested from a 4-day main-culture with CSF-1 + IL-2 (500 U/ml each), washed, and then incubated for 4 hours in different test media with Yac-1 as targets to test the cytotoxicity.

3.4.2 Detection of Perforin mRNA by Northern Blot Analysis

Northern blot analysis was used to investigate whether the RNA-preparations isolated from MP cells contained messenger RNA. For this purpose, as described under 2.14.1, the whole RNA from different MP cell samples were extracted, separated in gel, transferred on a N^+-nylon membrane and hybridized with ^{32}P-labeled cDNA probes. Three cDNA probes, specific complementary to a) mRNA for murine perforin [Lowrey et al. 1989]; b) mRNA for CSF-1 receptor [Rothwell & Rohrschneider 1987]; and c) mRNA for actin [Cleveland et al, 1980], were hybridized one after another with the

same membrane. The examined MP cell samples were as follows:

1. NK-inactive MP cells isolated from BM pre-culture with GM-CSF (2.10.3);
2. NK-like MP cells isolated from BM pre-culture with GM-CSF + IL-2;
3. NK-like MP cells in main-culture with CSF-1 + IL-2 for 3 days;
4. NK-like MP cells in main-culture with IL-2 for 3 days;
5. NK-like MP cells in main-culture with CSF-1 for 3 days;
6. NK-inactive MP cells in main-culture with IL-2 for 3 days.

As shown in **Figure 22**, the mRNA for perforin was found in the NK-like MP cells as well as in those cells after further culture with CSF-1 + IL-2 or IL-2 alone. This finding indicates a relationship between the expression of perforin mRNA and the cytotoxic function of the cells, because only the cells confirmed in cytotoxicity test (see **Tab. 6**) as lytic were positive for the perforin mRNA. All cell samples showed positive for c-fms, an important hint for the belonging of the cells to the macrophage series [Baccarini & Stanley 1990]. The actin bands served as controls, suggesting that from each cell sample the same amount of RNA was used.

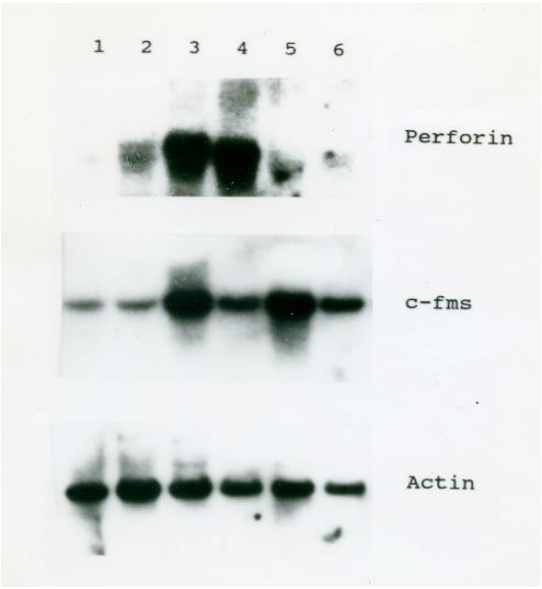


Fig. 22. Northern blot analysis for the expression of perforin mRNA by MP cells. The numbers 1-6 of RNA samples correlate to the cell samples 1-6 displayed above. The film was exposed for about 48 hours.

Tab. 6. ***Cytotoxicity against Yac-1 from Different MP Cell Samples*** [a]

No. [b]	**Cell Sample**	**Cytotoxicity（LU/10^7）**
	MP cells from BM pre-culture with：	
1.	GM-CSF 200 U/ml	0
2.	GM-CSF 200 U/ml + IL-2 100 U/ml	35
	NK-like MP cells in main-culture with：	
3.	CSF-1 500 U/ml + IL-2 500 U/ml	375
4.	IL-2 500 U/ml	380
5.	CSF-1 500 U/ml	0
	NK-inactive MP cells in main-culture with:	
6.	IL-2 500 U/ml	0

[a] The cells to be tested were cultivated in relevant cultures for 3 days. On day 4, when they were harvested for Northern blot analysis for the examination of perforin messenger RNA, a part of each cell sample was used respectively for the cytotoxicity test. The cytotoxicity test was carried out at 37°C for 4 hours. The lytic units (LU/10^7) were calculated as described under 2.12.2.

[b] The numbers 1-6 of cell samples correlate to the lane 1-6 shown in the outcome of Northern blot analysis (**Fig. 22**).

3.4.3 Detection of Perforin mRNA by *in situ* Hybridization

It was then further attempted, with the fixed cells on slides, to perform an *in situ*

Hybridization with cDNA for perforin mRNA to directly detect perforin mRNA in the cells. As described under 2.14.2, the cells to be examined, isolated from 4-day main-culture in CSF-l + IL-2 (500 U/ml each), were spun on microscopic slides. After they had been air-dried, they were fixed, permeabilized, and finally hybridized with DIG-labeled cDNA for perforin mRNA for 16-20 hours. A cytopreparation with normal macrophages served as a control. After the hybridization, the slides were washed in several steps (see 2.14.2.4) and then incubated with the anti-DIG antibody coupled to alkaline phosphatase. In the end, the hybridization was made visible by the addition of NBT + X-Phosphate, the color of which changes from yellow to blue when catalyzed by alkaline phosphatase. The dark blue stained cells were positive for perforin mRNA. **Figure 23** is the light microscopic image of the cells after the hybridization with DIG-labeled cDNA for perforin.

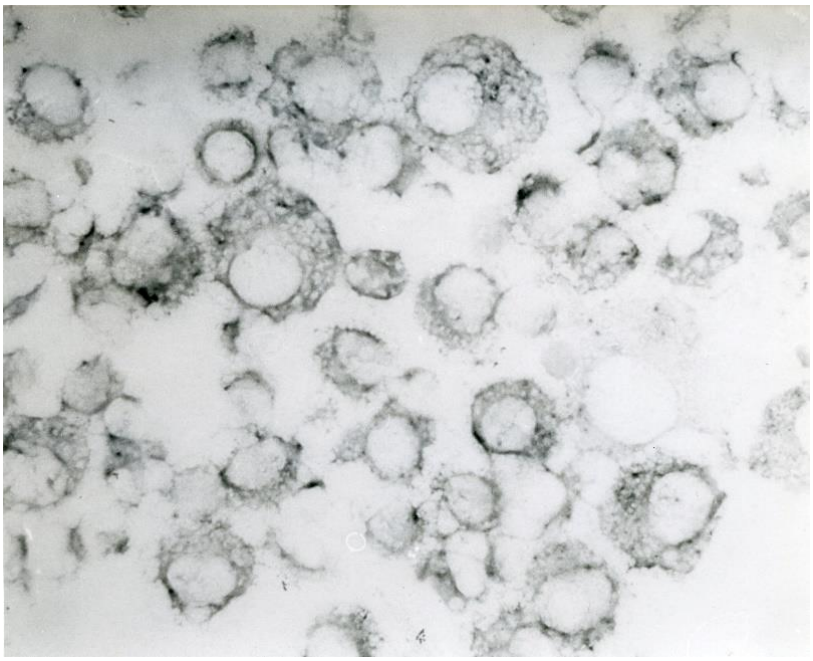


Fig. 23. Result of *in situ* hybridization with cDNA for perforin. The NK/LAK-like MP cells were isolated from a 4-day main-culture with CSF-l + IL-2 (500 U/ml each) and then *in situ* hybridized with DIG-labeled cDNA for perforin (see 2.14.2).

3.4.4 Detection of Perforin Proteins by Immunohistochemical Staining

Monoclonal antibody specific anti-mouse perforin was used for the immuno-histochemical staining to determine whether perforin proteins are present in the cells and where they are located. For this purpose, the cytopreparations of highly lytic effector cells from 4-day main-culture in CSF-l + IL-2 (500 U/ml each) were, as described under 2.11.5, prepared, fixed, incubated with mAb rat anti-mouse perforin and then with polyclonal antibody anti-rat IgG from goat coupled to alkaline phosphatase. Fast Red TR, a substrate for alkaline phosphatase [Dejong et al. 1985], was used for the coloring reaction. In **Figure 24** is to see that almost all cytoplasmatic granules are stained. In contrast, the cells that were agranular and NK-inactive were complete negative for this staining.

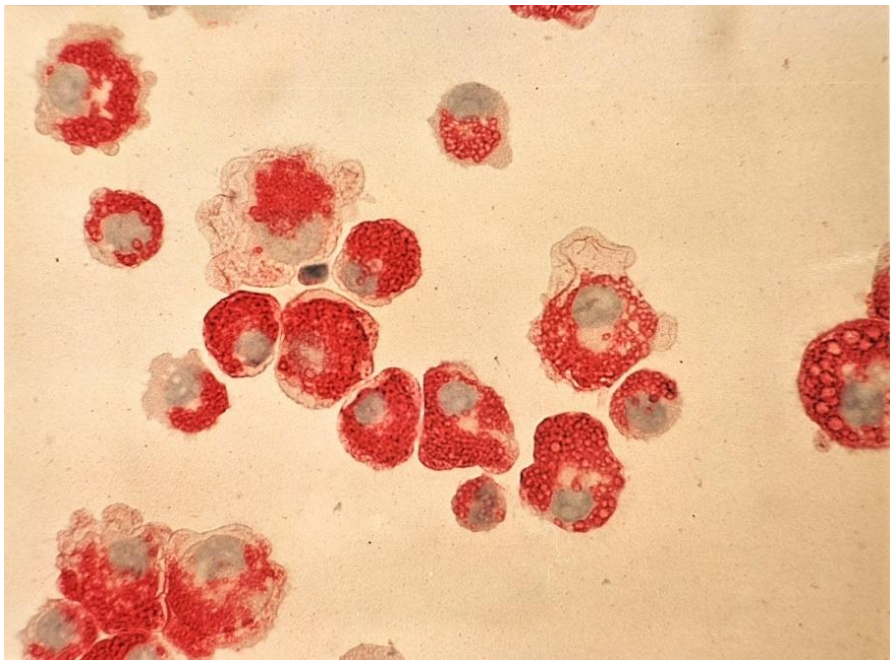


Fig. 24. Immunohistochemical staining with mAb anti-murine perforin. The NK-like MP cells isolated from the BM pre-culture were incubated in main-culture with CSF-l + IL-2 (500 U/ml each) for 4 days, and then immunohistochemically examined for perforin proteins with anti-murine perforin antibody (2.11.5).

3.5. Cytokine Release of NK-Active Macrophage Precursor Cells

In addition to cytotoxicity, NK cells perform another important function: they release cytokines. This happens when NK cells are stimulated by their target cells, by factors, or by a combination of both. The most commonly studied conditions for specific activation of NK cell are the following: 1) Incubation with target cells; 2) Incubation with target cells + IFN-γ; and 3) Incubation with IL-2 + ligands for CD16 Fc Receptor [Herman et al., 1986; Peters et al., 1986; Cuturi et al., 1989]. Furthermore, unspecific stimulators are used to stimulate NK cells, such as the protein kinase C activator phorbol ester PMA and Ca^{2+}-Ionophore [Paya et al., 1988; Anegon et al., 1988; Cuturi et al., 1989]. In the following experiments, the NK-active MP cells were treated under similar stimulation conditions. Yac-1, Yac-1 + IFN-γ, Yac-1 + IL-2, PMA (phorbol ester) as well as Ionomycin (Ca^{2+}-Ionophore) were used as stimulators to incubate with NK-like MP cells. Complete medium, P815, IFN-γ or IL-2 alone served thereby as controls. After incubation, the supernatants of the cultures were collected and then tested for cytokines. For comparison, the NK-inactive MP cells were treated under the same conditions and tested in parallel.

3.5.1. Release of Regulatory Factors after Stimulation with Target Cells and Cytokines

3.5.1.1 TNF-α

NK cells can release TNF by contact with target cells or target cells + IFN-γ [Peters et al. 1986]. Presumably, TNF is involved in the killing of target cells by NK cells through triggering the apoptosis effect or DNA fragmentation [Young et al. 1990]. In this experiment, it was to determine whether NK-like MP cells could also be activated by

addition of NK target cells to secrete TNF. Therefore, the effector cells (MP cells) were plated in 96-well microtiter plates with 1×10^5 cells/well and incubated respectively with Yac-1 (as target cells) as well as P815 (as control) alone or in combination with IFN-γ. The ratio of MP cells to tumor cells was 4：1. After about 20 hours, the supernatants of the cultures were collected and serially titrated on the TNF-sensitive L929 cells for determination of TNF-activity (see 2.13.7). In **Table 7** is to see that NK-like MP cells actively produced TNF after incubation with Yac-1. Supplement of IFN-γ led to a significant increase of TNF production. NK resistant P815 cells showed only very little effect on the TNF release of MP cells. With IFN-γ, the effect could be improved to some extent. In the experiment, in which the NK-inactive MP cells were used, neither Yac-1 or P815 alone nor the combination of tumor cells plus IFN-γ resulted in significant TNF production.

Tab. 7. ***Release of TNF from MP Cells after Stimulation by Tumor Cells***

20 h Incubation with	**NK-active MP** TNF（U/ml）		**NK-inactive MP** TNF（U/ml）
	no anti-TNFα	with anti-TNFα	
Complete medium	0		0
IFN-γ	0		0
Yac-1	32±1 [a]	0	1
Yac-1 + IFN-γ	58±2 [b]	0	2
P815	4±1	0	1
P815 + IFN-γ	8±1	0	2

MP cells：Tumor cells = 4：1　　IFN-γ: 500 U/ml

[a] $p < 0.001$　Yac-1 vs. P815　　[b] $p < 0.001$ Yac-1 + IFN-γ vs. P815 + IFN-γ

To identify the type of TNFs released from NK-like MP cells, an inhibition test was made with specific monoclonal anti-mouse TNF-α antibody (see 2.13.7), in which L929 cells were incubated with TNF-containing supernatants in the presence of anti-TNF-α. As shown in **Table 7**, the TNF activity was completely inhibited by anti-TNF-α. This indicates that NK-like MP cells release TNF-α.

3.5.1.2 IL-1

Since it was described that NK-sensitive (but not NK-resistant) tumor cells could stimulate NK cells to product IL-1 [Herman & Rabson 1986], NK-active and NK-inactive MP cells were incubated respectively with Yac-1 and P815 (both tumor cells do not produce IL-1) for 20 hours, and then the supernatants were examined for IL-1 activity. IL-1 activity was detected by colorimetric bioassay using the IL-1-dependent cell line D10.G4.1 (see 2.13.1). **Table 8** shows that the highest IL-1 production of NK-

Tab. 8. ***Release of IL-1 from MP Cells after Stimulation by Tumor Cells***

20 h Incubation with	**NK-active MP**		**NK-inactive MP**	
	$O.D._{570\ nm}$	IL-1 (U/ml)	$O.D._{570\ nm}$	IL-1 (U/ml)
complete medium	0.244±0.008 [b]	2	0.090±0.002	0
Yac-1	0.882±0.026 [a]	8	0.282±0.40 [c]	2
P815	0.242±0.022	2	0.066±0.015	0

MP cells：Tumor cells = 4：1

[a] $p < 0.001$ Yac-1 vs. P815

[b] $p < 0.001$ NK-active vs. NK-inactive

[c] $p < 0.001$ Yac-1 vs. complete medium

active MP cells was observed in the incubation with Yac-1. In contrast to NK-inactive MP cells, the NK-active cells were able to spontaneously secrete small amount of IL-1. P815 tumor cells, on the other hand, did not exhibit an IL-1-induction effect. In the incubation of NK-inactive MP cells with Yac-1, low IL-1 production was found.

3.5.1.3 IL-6

IL-6 also belongs to the inflammatory factors that can be released by NK cells. It was described that the activation of NK cells by IL-2 leads to strong IL-6 gen-expression [Vitolo et al., 1993]. In the following, the MP cells were activated respectively with IL-2, Yac-1, or a combination of both for 20 hours, and the supernatants were tested for their IL-6 content using the IL-6-dependent cell line 7TD1 (2.13.2). P815 tumor cells were not used in this test because they themselves are IL-6 producers. As shown in **Table 9**, both NK-active and NK-inactive MP cells were activated to release IL-6 when

Tab. 9. ***Release of IL-6 from MP Cells by Activation with IL-2 and Yac-1***

20 h Incubation with	**NK-active MP** IL-6（U/ml）	**NK-inactive MP** IL-6（U/ml）
Complete medium	20±0.4	0
IL-2	26±0.5	0
Yac-1	4000±80 [a]	258±25
Yac-1 + IL-2	7200±24 [b]	557±35

MP cells：Yac-1 = 4：1 IL-2: 500 U/ml

[a b] $p < 0.001$ NK-active vs. NK-inactive

stimulated by Yac-1 alone or Yac-1 + IL-2. However, the NK-active MP cells secreted about 10 times higher amount of IL-6 than the NK-inactive cells. Low spontaneous IL-6 release was observed in the NK-active MP cells, but not in the NK-inactive cells. In this experiment, the activation with IL-2 alone showed no significant influence on the IL-6 release of the cells.

3.5.1.4 IL-10

It is still not clear whether NK cells can produce IL-10 (also called Cytokine Synthesis Inhibitory Factor, CSIF). It is known that this relatively newly discovered factor can be released not only by Th2 (T helper 2) cells, B cells, but also by monocytes-macrophages [Den-Waal-Malefyt et al., 1991; Fiorentino et al., 1991b]. In the following experiments, the NK-active as well as the NK-inactive MP cells were incubated with a number of stimulators as shown in **Table 10**, and then IL-10 in the supernatants was examined by ELISA (2.12.5 and 2.13.3). It was found out that MP cells secreted IL-10 after incubation with Yac-1 (see **Tab. 10**). Yac-1 + IFN-γ stimulated both NK-active and NK-inactive MP cells to produce IL-10, however the NK-active cells were already able to secrete large amount of IL-10 after contacting with Yac-1 without IFN-γ. The NK-inactive cells were not able to do this. The addition of IL-2 did not lead to significant increase of IL-10 secretion from MP cells. A release of IL-10 from Yac-1 cells is excluded, because all supernatants from Yac-1 cells alone or with factors (IL-2, IFN-γ) were negative for IL-10. The P815 tumor cells did not stimulate MP cells to secrete IL-10.

Tab. 10. ***Release of IL-10 from MP Cells***

20 h Incubation with	**NK-active MP** IL-10（U/ml）	**NK-inactive MP** IL-10（U/ml）
Complete medium	0	0
IFN-γ	0	0
IL-2	0	0
Yac-1	22.0±2 [a]	2.5±0.4
Yac-1 + IFN-γ	37.3±2 [b]	19.4±1
Yac-1 + IL-2	25.9±2 [c]	2.6±0.5
P815	0	0
P815 + IFN-γ	0	0
P815 + IL-2	0	0

MP cells：Tumor cells = 4：1 IFN-γ: 500 U/ml IL-2: 500 U/ml

[a,b,c] $p < 0.001$ NK-active vs. NK-inactive

3.5.1.5 IFN-γ

IFN-γ production is one of the most important functional properties of NK cells, because IFN-γ shows a strong antiviral activity and works antimitotic on transformed cells (the cells infected by virus, translation notes). IFN-γ synthesis by NK cells takes place mainly after the activation by antigen plus IL-2 [Cuturi et al., 1989]. In the following experiments, NK-active and NK-inactive MP cells were incubated 20 h with

tumor cells alone or with tumor cells + IL-2. Afterwards, IFN-γ content in the supernatants were determined by ELISA (see 2.12.5 and 2.13.6). It was observed that the NK-active MP cells clearly differed functionally from the NK-inactive cells. In contrast to the NK-inactive MP cells, which never secrete IFN-γ under the tested conditions, the NK-active MP cells secreted IFN-γ after activation by a combination of tumor cells with IL-2 (**Tab. 11**). In this test, Yac-1 cells and P815 cells showed a similarly strong effect on the NK-active cells. IFN-γ release from NK-active MP cells can thus be stimulated by other cells than NK-target cells.

Tab. 11. ***Release of IFN-γ from MP Cells***

20 h Incubation with	**NK-active MP** IFN-γ（ng/ml）	**NK-inactive MP** IFN-γ（ng/ml）
Complete medium	0	0
IL-2	0	0
Yac-1	0	0
Yac-1 + IL-2	6.3±0.1	0
P815	0	0
P815 + IL-2	6.4± 0.2	0

MP cells：Tumor cells = 4：1　　IL-2: 500 U/ml

3.5.2. Release of Regulatory Factors after Stimulation with Phorbol Ester or Ca^{2+}-Ionophore

In the following tests, the MP cells in 24-well microtiter plates (1×10^6 cells in 2 ml medium/well) were activated respectively with PMA (0.2 µg/ml) and Ionomycin (1.2 µg/ml) for 20 hours. The cytokines released by the cells after the activation, such as TNF, IL-1, IL-6, IL-10 and IFN-γ, were determined either by colorimetrical bioassay or by ELISA. As shown in **Table 12**, activation of MP cells by the protein kinase C activator PMA did not induce the release of IL-10 or IFN-γ, nor did the activation by Ca^{2+}-ionophore ionomycin. The TNF test was made only with the samples activated by ionomycin (as PMA has a toxic effect on L929 cells). However, no TNF activity was observed in the tested supernatants. In contrast, the releases of IL-1 and IL-6 from MP cells could be activated by PMA or ionomycin. NK-active and NK-inactive cells behaved similarly in this study, although the NK-active MP cells produced more IL-6 than NK-inactive ones, especially after activation with ionomycin.

Tab. 12. ***Cytokine Release from MP Cells after Activation by PMA and Ionomycin***

Cytokine	NK-active MP		NK-inactive MP	
	PMA	Ionomycin	PMA	Ionomycin
TNF U/ml		0		0
IL-1 U/ml	6	13	2	6
IL-6 U/ml	620	2500	267	257
IL-10 U/ml	0	0	0	0
IFN-γ ng/ml	0	0	0	0

Duration of activation: 20 h PMA: 0.2 µg/ml Ionomycin: 1.2 µg/ml

3.5.3. Production of Hematopoietic Growth Factors

Reports about production of hematopoietic growth factors by NK cells have shown that after activation, mainly by IL-2, or IL-2 + NK-ligands, or phorbol ester (PMA) /Ca^{2+}-ionophore, m-RNA of GM-CSF, IL-3, as well as M-CSF in NK cells was expressed [Cuturi et al., 1989; Murphy et al., 1992]. These reports mentioned that detection of these growth factors by bioassays is difficult, because the supernatants to be tested could also contain proliferation-inhibitory factors such as TNF, IFN-γ. In the following experiments, the investigations for CSFs were only made with those supernatants which were free from TNF, IFN-γ, and IL-10. Only the supernatants obtained after incubation of MP cells with PMA, ionomycin, or IL-2 met this requirement (see **Tab. 10**, **11**, **12**). Since the LAK effector cells developed from NK-like MP cells (see under 3.3.) were similar to IL-2 cultivated NK cells described by other researchers, they were also tested in corresponding experiments.

3.5.3.1 GM-CSF and IL-3

The colorimetrical bioassay using the GM-CSF/IL-3 dependent cell line FDCP1 (2.9.2) was conducted as described under 2.13.5. The supernatants to be tested, collected from the cultures with NK-active or NK-inactive MP cells or LAK effector cells, were incubated with FDCP1 cells in microtiter plates. Complete medium served as control. In order to distinguish the IL-3 activity from the GM-CSF activity, specific anti-IL-3 antibody was pipetted into the appropriate test wells and the inhibition degree was tested. As shown in **Table 13**, GM-CSF/IL-3 activity was found only in the supernatants from NK- and LAK-active cells. Ionomycin stimulated only the IL-3-production from NK- and LAK-active cells, because the cell growth of FDCP-1 was completely inhibited by anti-IL-3 antibody. On the other hand, IL-2 activation stimulated apparently only the LAK cells to release GM-CSF.

Tab. 13. ***Release of GM-CSF or IL-3 from the Stimulated MP Cells***

Cells and Stimulators	O.D.570 nm		GM-CSF (U/ml)	IL-3 (U/ml)
	no anti-IL-3	with anti-IL-3		
1）NK-inactive MP:				
Complete medium	0.010±0.003		0	0
PMA	0.066±0.001		0	0
Ionomycin	0.096±0.004		0	0
IL-2	0.034±0.006		0	0
2) NK-active MP:				
Complete medium	0.072±0.002		0	0
PMA	0.098±0.000		0	0
Ionomycin	0.426±0.013 [a]	0.068±0.001	0	1.5
IL-2	0.082±0.007		0	0
3) LAK-active MP:				
Complete medium	0.036±0.000		0	0
PMA	0.097±0.004		0	0
Ionomycin	0.486±0.070 [b]	0.070±0.002	0	1.75
IL-2	0.488±0.004 [c]	0.430±0.032	16	0

Duration of incubation with stimulators: 20 h

PMA: 0.2 μg/ml Ionomycin: 1.2 μg/ml IL-2: 500 U/ml

[a] $p < 0.001$ Ionomycin vs. complete medium

[b] $p < 0.001$ Ionomycin vs. complete medium

[c] $p < 0.001$ Ionomycin vs. complete medium

3.5.3 M-CSF

To investigate M-CSF activity, soft-agar assay was used (see 2.12.6). The supernatants of MP cells that had been activated by PMA, ionomycin, or IL-2 were mixed with 0.35% agar and fresh BM cell suspension (1×10^5 cells/ml) in a ratio of 1 : 1 : 8 in 3.5 cm culture dishes. As a result, the supernatants were diluted 1 : 10. After a 7-day incubation, the colonies on the agar were counted under the light microscope. To determine the cell types in the colonies, the soft-agar disc with colonies were placed on slides, air dried, fixed, and finally stained by esterase double staining (2.11.3). The brownly stained cells were monocytes-macrophages [Li et al., 1973]. It was observed that the NK-active MP cells released colony-stimulating factor (CSF) after activation by PMA. Since all cells in the built colonies were stained brown after esterase double staining, M-CSF should be present in the supernatants. The induction effect of PMA on CSF-release was not found in NK-inactive MP cells.

Tab. 14. ***Production of M-CSF by MP Cells***

20 h Incubation with	**NK-active MP**		**NK-inactive MP**
	Colony number	CUF/ml	Colony number
Complete medium	0	0	0
PMA	28.5±11.2	285	0
Ionomycin	0	0	0
IL-2	0	0	0

PMA: 0.2 μg/ml Ionomycin: 1.2 μg/ml IL-2: 500 U/ml

To give an overlook about cytokine-release capability of MP cells, **Table 15** has been made. Data of NK-active and NK-inactive cells were presented separately in **Tab. 15.a** and **15.b** (see page 90).

Tab. 15.a ***A Short Summary of Cytokine Release from NK-Active MP Cells***

Stimulators	IL-1 (U/ml)	IL-3 (U/ml)	IL-6 (U/ml)	IL-10 (U/ml)	TNF-α (U/ml)	IFN-γ (ng/ml)	GM-CSF (U/ml)	M-CSF (U/ml)
unspecific:								
PMA	6	0	620	0		0	0	285
Ionomycin	13	1.5	2500	0	0	0	0	0
specific:								
Yac-1	8		4000	22	32	0		
Yac-1 + IFN-γ				37	58			
Yac-1 + IL-2			7200	24		6.3		
controls:								
C. medium*	2	0	20	0	0	0	0	0
IFN-γ				0	0			
IL-2		0	26	0		0	0	0
P815	2			0	4	0		
P815 + IFN-γ				0	8			
P815 + IL-2				0		6.4		

Tab. 15.b ***A Short Summary of Cytokine Release from NK-Inactive MP Cells***

Stimulators	IL-1 (U/ml)	IL-3 (U/ml)	IL-6 (U/ml)	IL-10 (U/ml)	TNF-α (U/ml)	IFN-γ (ng/ml)	GM-CSF (U/ml)	M-CSF (U/ml)
unspecific:								
PMA	2	0	267	0		0	0	0
Ionomycin	6	0	257	0	0	0	0	0
specific:								
Yac-1	2		268	3	1	0		
Yac-1 + IFN-γ				19	2			
Yac-1 + IL-2			557	3		0		
controls:								
C. medium*	0	0	0	0	0	0	0	0
IFN-γ				0	0			
IL-2		0	0	0		0	0	0
P815	0			0	1	0		
P815 + IFN-γ				0	2			
P815 + IL-2				0		0		

* C. medium: complete medium.

4. DISCUSSION

4.1. Are NK Cells the IL-2 Activated Macrophage Precursors?

Over the past 20 years (1973-1993, translation notes), scientists with strong interest have been searching for the origin of NK cells, for their lineage, and for their evolution from precursors to mature effector cells in order to learn more about and identify these cells, which are actively involved in the body's defense against tumors, viral and microbial infections. In 1977, Haller et al. showed by *in vivo* experiments in mice that the bone marrow is the site of origin of NK cells [Haller et al. 1977]. This result was later supplemented by Hackett et al. with the data from *in vitro* studies. They showed that the bone marrow environment is essential for the differentiation of NK cells [Hackett et al.1986]. Studies in the rat and human systems have later confirmed that the NK/LAK cells derived from bone marrow precursors [Sarneva et al. 1989; Keever et al. 1990]. All these data led to the conclusion that the NK/LAK effector cells develop from bone marrow precursors.

An open question in the NK/LAK research is still to which cell lineage the NK/LAK cells belong and what is the growth factor for these cells. Although IL-2 seems to be the most important factor for the NK/LAK cultures, the effect of IL-2 on the cells is stimulating rather for cytotoxicity than for proliferation. For example, Olabuenaga et al. reported as early as in 1983 that IL-2 alone is not sufficient for the growth of a cloned NK cell line [Olabuenaga et al. 1983]. Several years later, some research groups, who worked with the NK/LAK cells from spleen or from bone marrow culture, confirmed that for the proliferation of NK/LAK cells, another factor is required besides IL-2 [Gunji et al. 1989; Van-Den-Brink et al. 1990; Ayroldi et al. 1992]. This another factor has not yet been identified.

Since the growth factor for NK cells is unknown, it is in fact difficult with the help of *in vitro* culture, especially with the help of bone marrow culture to study the development of NK cells from the precursors to the effector cells. In the past five years (1989-1993, translation notes), almost all studies on the bone marrow precursors of NK/LAK effectors in rat, human and mouse were made only by means of “LTBMC” (long-term bone marrow culture) [Van-Den-Brink et al. 1990; Pollack et al. 1992; Sitnicka &. Hansson 1992; and Vecchini et al. 1993]. Under "LTBMC" conditions, the whole bone marrow cells were pre-cultured at a density of ca. 2×10^6 cells/ml in medium (RPMI 1640 + 5% FCS + 50 μM 2-mercaptoethanol) without exogenous factors to obtain an adherent stroma-cell layer. After a 4-week pre-culture, less than 3.5% of the cells had survived from the initial population [Vecchini et al. 1993]. Among the surviving cells were mostly adherent stroma cells and few nonadherent cells that were loosely attached to the stroma-cell layer. Then IL-2 was given into the culture. During a 3-6-day incubation with IL-2, the nonadherent cells developed first to NK- and further to LAK-effector cells, therefore they were seen as NK precursors [Sitnicka &. Hansson 1992]. In all these studies, the authors showed consistently that the stroma is necessary for the generation of NK cells in the bone marrow culture, and that under "LTBMC"-conditions, the development of precursors to NK cells requires a combination effect of IL-2 and a growth factor, which was built by the stroma [Ayroldi et al. 1992; Vecchini et al. 1993]. A concrete describing of this growth factor from stroma is not found in the literature.

"LTBMC " conditions have apparently the following disadvantages: (1) The culture lasts very long, thus it is in danger to be contaminated; (2) The stroma serves as a source of many cytokines, so that it is difficult to define a growth factor for NK precursors; and (3) The culture does not contain exogenously added factors in the first 4 weeks, this can result in a massive death of the stem cells, consequently the outcome of the NK

precursor cells can be remarkably decreased. Because of these problems, the "LTBMC" culture is not considered as practical or ideal bone marrow culture condition for the research of NK precursors.

The present work was based on the thoughts that the NK effector cells could be related to promonocytes [Lohmann-Matthes et al. 1979]. Instead of searching for the other growth factors, the myeloid growth factors, GM-CSF and CSF-1, were applied to stimulate the proliferation of bone marrow precursors for the NK effector cells. This had immediate success. Both the enriched NK precursor population from fresh bone marrow and the purified NK-active cell population isolated from a 3-day BM pre-culture are recognized as CSF-dependent cells in [^{3}H]-Thymidine incorporation test (**Fig. 2** and **Fig. 10**). In comparison to the "LTBMC"-culture, our bone marrow culture has following advantages: (1) Including the BM pre-culture and the main-culture, it needs only 3-7 days to induce the NK- and LAK-activities. Therefore, our culture is much shorter than the 4-week "LTBMC " culture; (2) Our bone marrow culture starts with well-known exogenous factors. For this reason, the running of the culture is easy to estimate and to control; (3) From our culture, a high cell yield is obtained. After 3 days of pre-culture, 3-5 $\times$ 10^6 precursor cells are harvested from one mouse, all of which can differentiate into NK/LAK effector cells. This cell number is 10 times higher than that obtained from "LTBMC" [Vecchini et al. 1993]; and (4) In our bone marrow culture with CSF plus IL-2, the stroma cells are not necessary for the production of NK/LAK activity. The culture can run with a pure cell population. This creates the prerequisite for tracing the development of the cells.

In the surface marker analysis of the cells from the "LTBMC" culture, it was often found that the nonadherent cells, which were valued as NK precursors, had various phenotypes. For example, Sitnicka et al. reported that in the "LTBMC" culture the non-

adherent cells were partly positive for the T cell marker CD3 [Sitnicka & Hansson 1992]. According to the definition for NK cells, CD3 should not be expressed by NK cells [Fitzgerald-Bocarsly et al. 1988], so the discovery of $CD3^+$ cells can only imply that the nonadherent population in the "LTBMC" culture is composed of heterogeneous cells. For this reason, it is not correct to call the nonadherent cells in the "LTBMC" culture a NK precursor population. So far, only one surface marker on the bone marrow precursor cells for the NK/LAK effectors has been determined, namely the specific marker for NK cells, i.e. NK-1.1 in the mouse [Hackett et al. 1986; Vecchini et al. 1993]. From this point of view, the precursor population isolated from our culture, in comparison to those from "LTBMC", is obviously in better condition in term of their expression of NK-1.1 (more than 95% cells positive, see **Fig. 9**) and their purity (complete negative for T, B cell markers, see **Fig. 9**).

The NK-1.1 positive bone marrow precursors from our culture turned out to be bipotent cells, they differentiated quickly to active NK/LAK effectors by IL-2 activation, but on the other hand, in absence of IL-2 and under the influence of CSF-1, they could mature into $F4/80^+$ macrophages (**Fig. 14** and **15**). This second property they have is in common with that in the literature defined bone marrow precursors for macrophages [Crocker & Gordon 1985]. The presence of NK-1.1 marker on mature macrophages (see **Fig. 15**) indicates to a common origin of the two cell types. Recently, Koo et al also reported that the F4/80 positive macrophages from mouse carry the NK-1.1 marker [Koo et al. 1991].

For 15 years (1979-1994, translation notes), the relationship between the cells from the macrophage system and the NK effector cells has been discussed [Lohmann-Matthes et al. 1979; Decker et al. 1986; and Baccarini et al. 1985, 1986, 1988, 1989]. Although

it has long been known that NK cells carry a typical myeloid marker Mac-1 [Zarling & Kung 1980; Tai & Werner 1980; Ortaldo et al. 1981], many authors assume that the NK cells belong to the lymphocytes. The main reason for this is that the production of NK activity is dependent on IL-2, and IL-2 is a well-known growth factor specific for lymphocytes [Herberman et al. 1987]. As mentioned above, to the NK cells, IL-2 is not responsible for the cell proliferation but for the cytotoxic function [Gunji et al. 1989; Van-den-Brink et al. 1990; Ayroldi et al. 1992]. Furthermore, the expression of IL-2 receptor on monocytes and granulocytes (see Introduction 1.2) is an indication that the effect of IL-2 is not specifically limited to lymphocytes. The IL-2 requirement of the NK/LAK effector cells is not evidence that the NK/LAK cells are lymphocytes.

In this work, a not yet described but important phenomenon was observed in the development of bone marrow macrophage precursors, that is: when these macrophage precursors of bone marrow meet the IL-2 stimulating signal at early stage, they will be potent to differentiate into NK/LAK effectors. As these effector cells exhibit morphologically, phenotypically, functionally as well as cyto-chemically all typical characteristics of NK cells, we have the opinion that these IL-2 activated macrophage precursor cells are the NK cells. Although our BM culture does not start with the whole bone marrow cells, but only with the light Percoll fraction, it was verified by the IL-2 stimulation test that the cells in the heavy Percoll fractions hardly contained any NK precursor cells (see **Tab. l**). Silvennoinen et al. showed likewise that the bone marrow precursors of NK cells are found only in the light Percoll fractions [Silvennoinen et al. 1986].

For the abovementioned reasons, it can be assumed that the NK/LAK effector cells described by other authors originate from the bone marrow precursors that can differentiate either by IL-2 activation into NK/LAK cells or by CSF-1 stimulation into

macrophages. Our results are partly compatible with those from Sitnicka et al., these authors showed by the experiments from the "LTBMC"-culture that the BM precursors for NK/LAK cells differentiated into granulocytes-macrophages under the influence of GM-CSF alone [Sitnicka & Hansson 1992]. We agree on this: that there is a common precursor for the NK cells and the myeloid cells in bone marrow. However, the stage of the precursors is defined differently by us. Sitnicka and Hasson have the opinion that these precursors are still the pluripotent stem cells, which can differentiate either into lymphoid or into myeloid cells. But our data showed that these precursor cells have already entered into the myeloid line, because the lymphocyte growth factor IL-2 could not stimulate the cells to proliferate (see **Fig. 2** and **10**) or to differentiate into $CD3^+$ T cells (see **Fig. 20**).

4.2. Differentiation of Bone Marrow Macrophage Precursors under the Influence of CSF-1 and IL-2

Based on the data of this work, a hypothesis about the differentiation possibilities of the macrophage precursors of bone marrow is proposed as shown in **Fig. 25**:

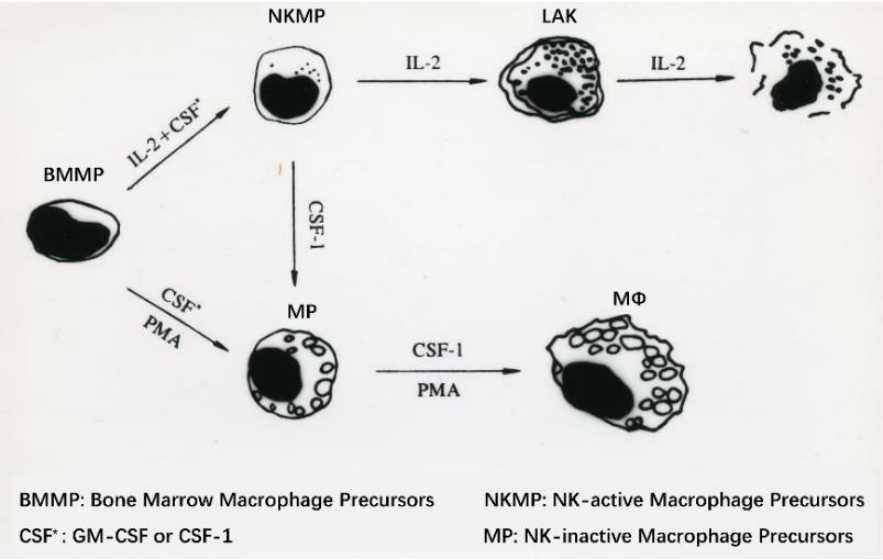
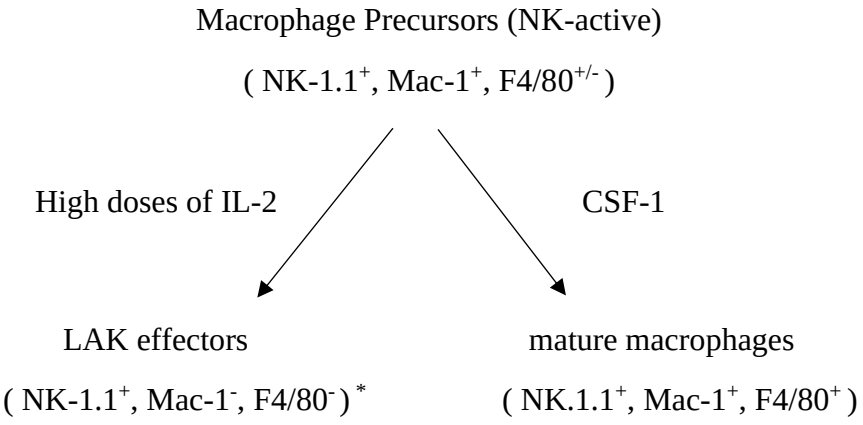


Fig. 25. A hypothesis about the differentiation possibilities of the macrophage precursors from bone marrow.

During the differentiation processes, the phenotype of the MP cells changes depending on the culture conditions. Among the monoclonal anti surface marker antibodies tested, NK-1.1 was the only common surface marker for all differentiation states of the MP cells. F4/80, a typical marker for the mature macrophages [Anstyn & Gordon 1981], could only be weakly detected on the NK-active cells but not on the LAK-active cells (see **Fig. 9** and **20**). Likewise, an ever more obvious decline of the expression of Mac-1 was observed in the development of NK-active to LAK-active cells (**Fig. 9** and **20**). In conclusion, it can be said that under the influence of IL-2 or CSF-1, the phenotype of BM macrophage precursors develops as follow:



Our observation, that in the LAK induction with high dose IL-2 the MP cells lose their surface markers Mac-1 and F4/80, agrees with the results from other researchers. Grimm et al. found in the LAK culture that the LAK effector cells are negative for Mac-1 marker [Grimm et al. 1982]. Parr et al. described that the NK/LAK-like cells containing a lot of perforins do not express F4/80 marker [Parr et al. 1990]. How such

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* Translation notes: In late 1994, we (Uwe Pohler and I) found that: The MP derived LAK effectors at this state became strongly positive for B220 and Thy-1.2 and remained negative for CD3 and CD8. See the additional Figures 3 and 4 on page 127 (not shown in the original dissertation).

cell markers are downregulated by IL-2 stimulation remains to be investigated.

This work shows that the effect of IL-2 on the macrophage precursors takes place at the gene level. In the MP cells that had been activated by IL-2, perforin messenger-RNA was found (**Fig. 22** Lane 2, 3, 4), which was not expressed constitutively by the normal MP cells (only cultivated with CSF, see **Fig. 22** Lane 1). The activation of the gene to synthesize the perforin messenger RNA appears to be caused by an early IL-2-stimulation, because the MP cells, which were precultured in CSF alone only for 3 days, were no longer able to synthesize the perforin-mRNA under the stimulation with IL-2 (see **Fig. 22** Lane 6). The data from the NK activity test also confirmed that an early supplement of IL-2 is essential for the NK induction (see **Fig. 4**). When IL-2 was first supplied 24 hours later into a pre-culture with CSF alone, the induction of NK activity was dramatically reduced; when IL-2 was first supplied 48 hours later, then no more induction was observed. This can be explained by the fact that the maturation process for macrophage development reduces the perforin production capacity of MP cells. Recently Ayroldi et al. have reported that the IL-2 induced NK development in bone marrow cultures was inhibited by PMA [Ayroldi et al. 1993]. The present work observed that PMA accelerates the mature process of precursor cells to macrophages (see **Fig. 6**). So, it can be assumed that PMA prevents the induction of NK activity by rapidly driving the young bone marrow precursors, which can react with IL-2, to a more mature stage. Therefore, despite the presence of IL-2, the cells are no longer able to synthesize perforin, the presence of which is essential for the NK activity. Further studies are needed to determine at what stage a precursor can no longer be stimulated to produce perforin.

When IL-2 was withdrawn from the culture, both perforin-mRNA in the cells (see **Fig. 22** Lane 5) and the NK activity (**Tab. 6**. No. 5) disappeared. So, the cytotoxicity of MP

cells against NK target cells (Yac-1) is strictly IL-2-dependent. The findings of the present study bring a new aspect to the long-standing controversial question of whether the NK/LAK cells belong to lymphocytes or to the macrophage system. Many authors presented data about the IL-2-dependence of NK/LAK activity to prove that the NK/LAK effector cells belong to lymphocytes. In addition, it has been shown that no NK cytotoxicity is found in the culture only with CSF-1 [Ortaldo et al. 1984; Koo & Manyak 1986]. Thus, they assumed that the cells of the macrophage system could not have NK activity. Although our data are in consistent with these results, but we come to different conclusions. Since the macrophage precursors require IL-2 to synthesize the perforin, the NK activity that relies on the presence of perforin is IL-2-dependent. On the other hand, CSF-1 only exerts a stimulating effect on cell growth, so it is to be expected that the cell culture with CSF-1 alone does not lead to NK activity. In previous studies about the similarities between promonocytes and NK cells, the important role of IL-2 for the induction of NK activity in macrophage precursors was not recognized. The bone marrow cultures at that time were performed with whole bone marrow cells in L cell-conditioned medium [Lohmann-Matthes et al. 1979; Baccarini et al. 1985, 1988]. The only known factor in the culture was CSF-1. Since recombinant CSF-1 is applied in the BM culture and the cultures start with the fractionated BM cells, NK activity in the cultures is not to be observed without addition of IL-2. This indicates that in the previous studies, the BM cultures probably produced themselves the NK activity stimulating factors, such as IL-2. The effect of IL-2 on macrophage precursors found in the present study could contribute an important complement to the differentiation theory of macrophage precursors.

IL-2 can also strongly influence the cytokine production capacity of MP cells. In this work, for example, it was observed that the IL-2-activated, NK-active MP cells secreted IFN-γ, while the normal, NK-inactive MP cells did not produce IFN-γ (**Tab. 11**). The

influence of IL-2 on the cells in early stage seems very important, because in the cytokine induction experiments, the same stimulator (tumor cells + IL-2) could stimulate the NK-active MP cells to release IFN-γ, but not the normal MP cells. It is well-known that IFN-γ is produced by activated T cells [Trinchieri et al. 1985] and NK cells [Michael et al. 1989], but not by monocytes-macrophages. Since our NK-active MP cell population was free from T cells (see **Fig. 9**), it is excluded that the IFN-γ found in this study was released by contaminated T cells. Moreover, we have observed that the IL-2 activated, NK-active MP cells are much more active in the productions of TNF (**Tab. 7)**, IL-6 (**Tab. 9**), IL-10 (**Tab. 10**), as well as colony-stimulating factors (**Tab. 13**, **14**) than the normal, NK-inactive MP cells.

Active cytokine production, especially CSF release of NK/LAK cells, suggests that the long-standing controversy over the proliferation effect of IL-2 on NK/LAK cells is likely due to one or more IL-2 induced factor(s). In 1989, Gunji et al. observed in an *in vitro* culture with the NK/LAK cells from spleen that except IL-2 also a "conditioned medium" derived from a 3-day pre-culture of the same cells with IL-2 (1000 U/ml) had to be added into the culture to induce the proliferation of NK/LAK cells. Based on our data (see **Tab. 13** LAK-active) and those from other laboratories [Cuturi et al. 1989], which show that IL-2 stimulates the NK/LAK effectors for CSF-synthesis, it can be assumed that this "conditioned medium" probably contains the colony-stimulating factors, such as GM-CSF, CSF-1, or IL-3, which are responsible for the proliferation of NK/LAK cells.

The data shown in the present work on the ability of NK-active MP cells to produce cytokines are almost consistent with those from the research of NK cells (see Introduction 1.5). The MP cells activated by IL-2 were obviously more sensitive to the

stimulation by NK target cells (Yac-1) than the normal, NK-inactive MP cells in terms of their TNF-, IL-1-, and IL-10-production (see **Tab. 7**, **8**, **10**). The results from the IL-10-investigation indicate that the MP cells are able to recognize the Yac-1 cells totally independent of IL-2 stimulation, because when stimulated with Yac-1 and P815 (see **Tab. 10**), both NK-active and NK-inactive MP cells responded only specifically to Yac-1 cells for IL-10 release. IL-10 is a relatively newly discovered factor that exercises both inhibitory effects on Th1 (T helper 1) cells and monocytes-macrophages [Fiorentino et al. 1991a and b; De-Waal-Malefyt et al. 1991, 1993] and stimulating effects on B cells [Go et al. 1990; Defrance et al. 1992], cytotoxic T cells [Chen & Zlotnik 1991], as well as mast cells [Thompson-Snipes et al. 1991]. The significance of this IL-10 release from MP cells is still unclear.

4.3. *in vivo* Application of Cultured LAK Cells and Analysis of the Cause of the Side Effects of Immunotherapy with LAK Cells plus High Dose IL-2

A few years ago (in 1985, translation notes), it was reported that immunotherapy with *in vitro* cultivated NK/LAK cells led to a regression of tumors [Lafreniere & Rosenberg 1985]. Unfortunately, there is still no effective culture condition available for a rapid *in vitro* expansion of NK/LAK cells. From the present work, a new *in vitro* culture condition, consisting of CSF as growth factor and IL-2 as cytotoxicity-stimulating factor, may emerge for the cultivation of NK/LAK effectors. In the immunotherapy, the *in vitro* cultivated LAK cells were often used with high dose IL-2 (as LAK activity stimulating factor). Thereby both regression of tumors and strong toxic side effects were observed, for example "vascular leak syndrome" [Rosenberg et al. 1985; Lotze et al. 1986; Rosenstein et al. 1986; and Peace et al. 1989]. The reason for this is still not explained. The present work has observed that high dose IL-2 provokes a significant increase of cytolytic perforins in NK/LAK effector cells (**Fig. 18** and **24**), and that the LAK cells filled with a lot of perforin granules finally fragment and so a release of

perforin from the LAK cells takes place (**Fig. 19**). This could be a reason for the toxic side effects. On the other hand, our results show that IL-2 leads to an increase of the IL-6 release (**Tab. 9**). As mentioned in Introduction 1.4, IL-6 has a synergistic effect on perforin synthesis induced by IL-2, so it is imaginable that perforin augmentation in LAK cells runs even more effective and faster due to the presence of IL-6. As a result, decomposition of LAK cells happens earlier in the body. For these reasons, it does not appear to be recommendable to apply high dose IL-2 together with LAK cells *in vivo*. The other side effects could be caused by the cytokines released from the NK/LAK cells. So, the cytokine production data of this work could be useful for the *in vivo* application of NK/LAK-active cells to avoid some side effects.

4.4. Comparison of the Properties of the Unstimulated Macrophage Precursors, the IL-2 stimulated Macrophage Precursors, and the NK/LAK Cells Described in the Literature.

Based on the results of the present work, the most important characteristic properties of both types of MP cells, namely: the normal (NK-inactive) and the NK-active macrophage precursors, are presented in summary in **Table 16** (see next page) and shown in comparison with the NK/LAK cells described in the literature.

Tab. 16. ***Comparison of Properties of MP Cells with Those of NK/LAK Cells***

Properties	MP Cells			NK/LAK Cells Described in Literature
	normal	NK-active	LAK-active	
1）General:				
Size	16-20 μm	16-20 μm	20-25 μm	16-20/20-25μm
N/C* Ratio	high	high	middle	high/middle
Adherence	-	-	+	-/+
Perforin Granules	-	+	++	+/++
2）Surface marker:				
Mac-1	+	+	-	+/-
NK-1.1	+	+	+	+/+
F4/80	weak	weak	-	?/-
3）Cytotoxicity:				
against Yac-1	-	+	++	+/++
against P815	-	-	++	-/++
4）Cytokine Release:				
IL-1	+	+		+
IL-3	-	+	+	+
IL-6	+	++		++
IL-10	+	++		
TNF-α	+	++		++
IFN-γ	-	+		+
GM-CSF	-	-	+	+
M-CSF	-	+		+

* N/C: Nucleus /Cytoplasm

The results of this dissertation suggest that the bone marrow precursor cells of macrophage systems can differentiate into cytolytic NK/LAK effector cells.

5. SUMMARY

In the present dissertation, a practical bone marrow culture condition for expanding the NK/LAK effector cells was established. This bone marrow culture contains CSF (CSF-1 or GM-CSF) as growth factor and IL-2 as NK/LAK activity stimulating factor, and can produce within 3-6 days approximately 10 times more NK/LAK effector cells than the nowadays commonly used LTBMC ("long-term bone marrow culture"), which normally lasts 4 weeks and was described as the only suitable bone marrow culture condition for NK/LAK cells [Vecchini et al. 1993].

The NK-active cells isolated from the bone marrow culture with CSF + IL-2 showed a LGL (large-granular lymphocyte)-like morphology and could be purified to > 90%. The phenotype (surface antigen profile) of the purified cells was positive for Mac-1 and NK-1.1 and negative for all typical T and B cell markers, such as Lyt-1, Lyt-2 (CD8), L3T4 (CD4) and B220. These NK-1.1 positive, NK-active cells proliferated in the presence of CSF (CSF-1 or GM-CSF), but not of IL-2, and differentiated within 24 hours into mature macrophages under the influence of PMA (phobol-12-myristate-13-acetate) that stimulates the differentiation. Therefore, they were identified as macrophage precursors.

The NK-active macrophage precursors could maintain their cytotoxicity in the presence of IL-2. When IL-2 was withdrawn from the culture, the cytotoxicity was no more detectable on next day. On the other hand, these NK-active macrophage precursors acquired LAK activity (lytic to both NK-sensitive and NK-resistant target cells) when they were cultivated with high dose IL-2. The phenotype of LAK-active cells (NK-1.1^{+}, CD3^{-}, Mac-1^{-} and F4/80^{-}) corresponded to the LAK cells described in the literature. The cytolytic activity of macrophage precursors stimulated by IL-2 based on a perforin mediated mechanism.

The investigation on cytokine release showed that the IL-2 stimulated, NK-active macrophage precursor cells were also active cytokine producers. They release significantly higher amount of IL-1, IL-6, IL-10 and TNF-α than the unstimulated, NK-inactive (normal) macrophage precursors. Furthermore, IFN-γ, colony-stimulating factors such as GM-CSF, M-CSF and IL-3 were secreted by IL-2 stimulated macrophage precursor cells, that was not observed in normal macrophage precursor cells. This spectrum of released cytokines matches the cytokine-pattern secreted by the NK/LAK cells described in the literature.

In summary, the results of this study show that by IL-2 activation the macrophage precursors of the bone marrow can develop into cells with typical features of NK/LAK cells.

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Resume

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9.1964-7.1969	Attending primary school in Chongqing
9.1969-2.1975	Attending middle school in Chongqing
8.1975-1.1978	Service in countryside in Ba County of Chongqing
3.1978-1.1982	Study for Biology at Sichuan University
2.1982-7.1983	Teaching assistant in the Biochemical Lab of Department of Earth-chemistry of Southwest-China Agriculture University
8.1983-10.1986	Research assistant in the Biochemistry Lab of Chengdu Biology Institute of Chinese Academy of Sciences
11.1986-11.1987	Scholarship for work in the Department of Immunobiology (Leading Professor M. -L. Lohmann-Matthes) of Fraunhofer-Institutes for Toxicology in Hannover
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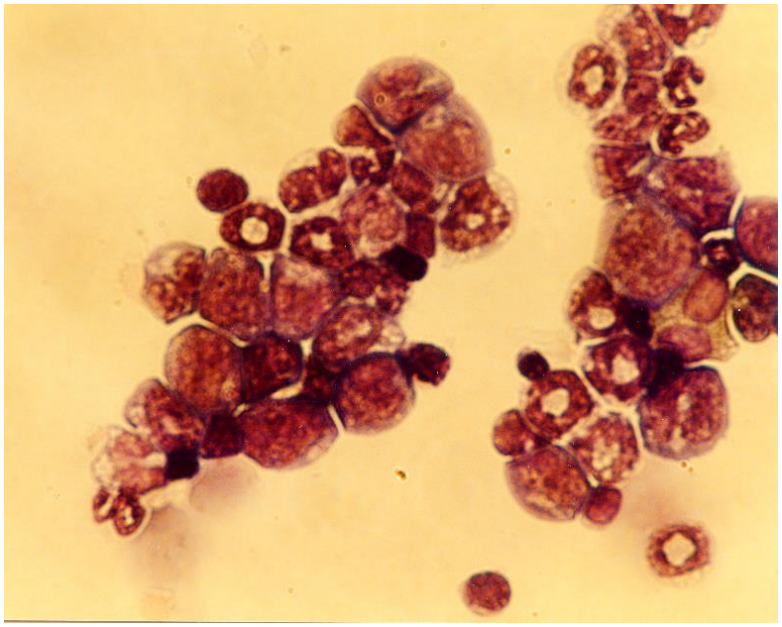
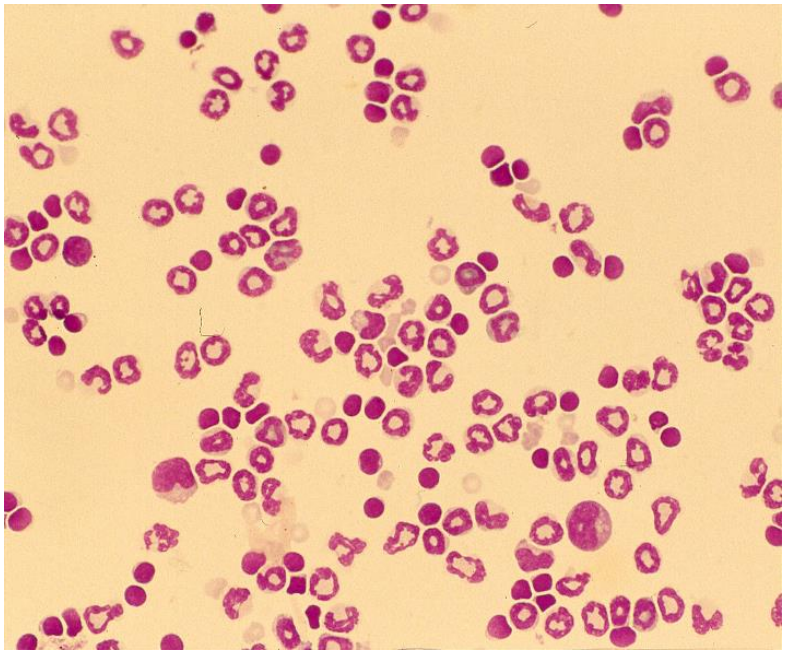
1.2023-12.2023	Translating this doctoral dissertation from German into Chinese and English

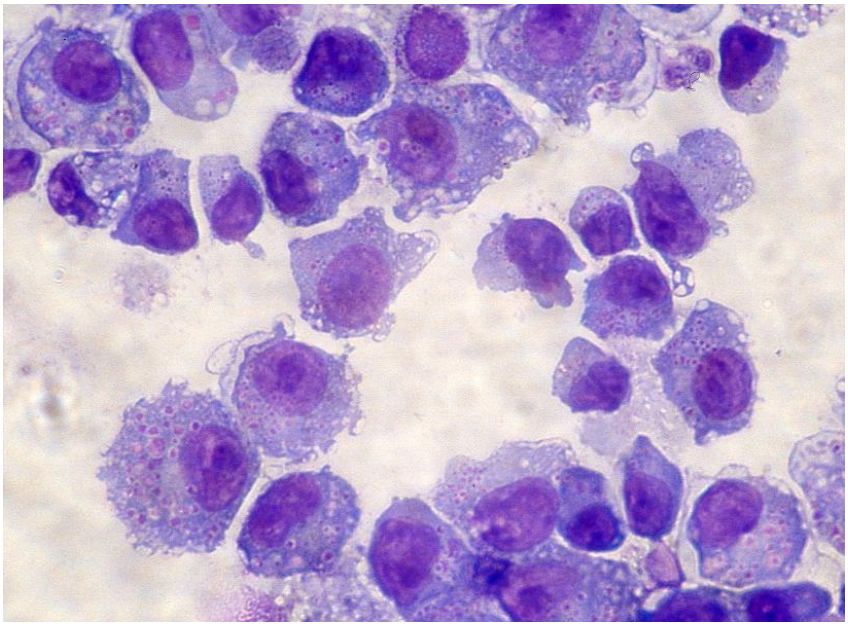
*Below the dotted line, the content was added by the end of 2023 for the English version of this dissertation.



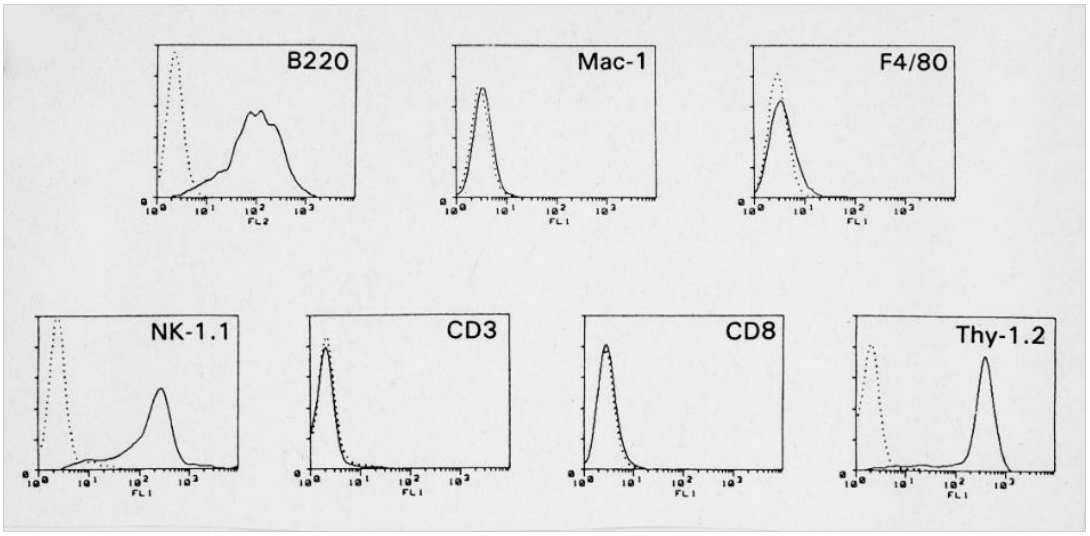
Additional Fig. 1. The morphology of murine BM cells in the light Percoll fraction (32%/52%). Most of the cells were unidentifiable precursors.



Additional Fig. 2. The morphology of murine BM cells in the heavy Percoll fraction (52%/55%). Little unidentifiable precursors could be found (the amplification is smaller than the additional figure 1).



Additional Fig. 3. The morphology of murine BM macrophage precursors cultivated in LAK culture (IL-2 500U/ml + CSF 50U/ml) for 5 days. The cells contained a lot of cytolytic granules.



Additional Fig. 4. The phenotype of murine BM macrophage precursors cultivated in LAK culture for 5 days. At this time, the cells became positive for B220 and Thy-1.2, both of which had not been previously expressed. The cells kept NK1.1 marker, but negative for Mac-1, F4/80, CD3 and CD8.

The End

(English version finished on Jan. 20, 2024)