IMMUNOMODULATION IN GRAM-NEGATIVE SEPTICEMIA

LIPOSOME-ENCAPSULATED MTPPE AND IFN-γ IN *KLEBSIELLA PNEUMONIAE* INFECTION IN MICE, EFFICACY AND MECHANISMS

TIMO L.M. TEN HAGEN

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Immunomodulation in Gram-negative septicemia:
Liposome-encapsulated MTPPE and IFN–γ in *Klebsiella pneumoniae* infection in mice, efficacy and mechanisms by Timo L.M. ten Hagen
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IMMUNOMODULATIE BIJ GRAM-NEGATIEVE SEPSIS

LIPOSOMAAL INGEKAPSELDE MTPPE EN IFN-γ IN *KLEBSIELLA PNEUMONIAE* INFECTIE IN DE MUIS, EFFECTIVITEIT EN MECHANISMEN.

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CONTENTS

CHAPTER ONE

Aim of the Study: A short Introduction into Previous Findings and Good Intentions Resulting From That.

Timo L.M. ten Hagen

The effect of MTPPE and IFN–γ, free or liposome-encapsulated, on host defense has been described in a large number of studies. An extensive elaboration on application of muramyl peptides (such as MDP and MTPPE) and IFN–γ, both in free form as well as liposome-encapsulated, in infections is given in the General Discussion. Many different infection types, both intracellular and extracellular, Gram-negative and Gram-positive bacterial, viral and protozoal infections are used. In our laboratory we focussed predominantly on a model of Gram-negative septicemia caused by *Klebsiella pneumoniae* to study the effect of MTPPE and IFN–γ (especially liposome-encapsulated) on the antimicrobial host defense. As this type of infection is difficult to treat with antibiotics and life-threatening in the immunodeficient host, effective activation of the antimicrobial host defence may be of great importance. In the infection model used in the studies described in this thesis *K. pneumoniae* organisms are injected intraperitoneally in mice, resulting in multiplication of the bacteria and appearance in the blood. Eventually all mice die due to septicemia.

Good efficacy is obtained with above mentioned immunomodulators in experimental infections, and this way of treatment is believed to be of great value in fighting severe and life threatening infections in immunodeficient hosts. However, only little information is available on the mechanism of host defense activation by these agents. Also studies on side effects and problems which might be encountered particulary when immunomodulators are used to fight infections in immunodeficient patients are limited. Previous studies performed in our laboratory with MTPPE (both free and liposome-encapsulated), in mice with *K. pneumoniae* septicemia, showed that the infection was effectively treated with the immunomodulator. In this thesis this model was used to further study the potency of liposome-encapsulated MTPPE and IFN–γ or the combination of MTPPE and IFN–γ encapsulated in liposomes. In addition, activation of host defence by the immunomodulators was examined mechanistically. In these studies immunocompetent mice were used as well as mice immunodeficient in various ways.

Previous findings in our laboratory with immunomodulators.

Administration of MTPPE prophylactically is effective in the model of *K. pneumoniae* septicemia in mice. Liposomal encapsulation of MTPPE (LE-MTPPE) resulted in 2-fold increase in potency of the immunomodulators in this model. Administration of the immunomodulators therapeutically appeared not effective or even resulted in increased death rates. However, intensification of prophylactic treatment by repeated dosages improved survival dramatically. With five dosages of LE-MTPPE alone a survival of approximately 85% was reached compared with 50% in the single dose treatment. These results favor multiple prophylactic treatments with the immunomodulator.

In the same *K. pneumoniae* infection model it was shown that blocking leukocyte recruitment with cyclophosphamide resulted in significant reduction in potency of LE-MTPPE to enhance antimicrobial host defense. However, host defense activation was completely abrogated when mice were depleted of their tissue macrophages, by intravenous injection of liposome-encapsulated dichloromethylene diphosphonate, before LE-MTPPE was administered. Taken together these observations demonstrate that leukocyte recruitment is very important, but that tissue macrophages are essential in host defense activation by liposomal MTPPE. These data are in agreement with results obtained with different mouse strains. It appeared that efficacy of treatment with MTPPE of a *K.*

pneumoniae infection depended on the strain of mice used in the infection model. This correlated with the capacity of the mouse strain to respond to immunomodulation with leukocyte recruitment. From these results it can be concluded that leukocyte recruitment induced by MTPPE is essential for MTPPE treatment to be effective.

In vitro studies with *L. monocytogenes*-infected macrophages in culture demonstrated that exposure of macrophages to MTPPE or IFN–γ or combination resulted in killing of intracellular microorganisms by infected cells. Also treatment with these agents in vivo is effective in mice infected with *L. monocytogenes*. From these data it was speculated that direct macrophage activation is involved in vivo in activation of host defense by immunomodulators. Taken together, in vivo granulocyte and macrophage depletion studies as well as in vitro studies reveal that both cell-types are important in the observed antimicrobial host defence activation upon immunomodulation. However, to which extent is still unclear.

Studies performed on biodistribution of liposomes demonstrated that liposomes predominantly localize in liver and spleen. These organs are known to have a very active mononuclear phagocyte system (MPS). High uptake of liposome by MPS cells (tissue macrophages) would explain these results. When both liposomes and bacteria were injected intravenously it appeared that liposome uptake did not correlate with bacteria uptake. Macrophages containing liposomes did not necessarily have intracellular bacteria and vice versa. It was concluded that probably macrophages which did take up liposomes are activated to secrete signal peptides which in turn activate the bacteria loaded macrophages. These findings are supported by observation that LE-MTPPE has to be given at least 6 hours before infection to be effective. These results might indicate that host defense activation resulting in increased resistance towards infection does not solely result from macrophage activation but also other mechanisms must be involved.

The observed increase in potency of MTPPE when liposome-encapsulated to stimulate host defense in the *K. pneumoniae* infection model was also observed in an experimental infection model utilizing *L. monocytogenes*. Moreover, dramatic augmentation of host defense to *L. monocytogenes* infection was also seen when IFN–γ was encapsulated in liposomes when compared with free IFN–γ. In vitro experiments on cultured macrophages infected with *L. monocytogenes* demonstrated synergy between MTPPE and IFN–γ in killing of the intracellular bacteria. Similar results could be obtained in vivo in mice infected with *L. monocytogenes* when the agents were co-encapsulated into liposomes. From these findings it can be concluded that liposomal encapsulated immunomodulators are preferred over agents in the free form when applied in vivo. In addition, synergy can be obtained in vivo when the immunomodulators are co-encapsulated in liposomes.

Aim of the study: emphasis on immunomodulation in experimental *K. pneumoniae* septicemia.

The aim of this thesis was to study the activity of liposomal MTPPE and IFN–γ in a *K. pneumoniae* septicemia model and to achieve insight in the mechanisms behind immunomodulator-induced host activation. To this end several experimental set-ups were designed and elaborated.

(1) Multiple dosages and co-encapsulation of MTPPE and IFN–γ was applied to find

best possible treatment in above mentioned infection. Thereto survival tests were performed. In addition, activity of the agents was studied in more detail on macrophages in vitro.

(2) Not only macrophages but also other cells are involved in host defense augmentation by immunomodulators. Studies were performed to examine involvement of T-cells and T-cell derived cytokines in immunomodulation. Also changes in T-cell profile were studied in mice upon administration of liposomal MTPPE and IFN–γ. T-cells were studied because these cells are known to have important functions in macrophage activation.

(3) Blocking of bone marrow activity, resulting in inability of immunomodulators to induce leukocyte recruitment, demonstrated that migration of leukocytes to blood or infectious site is important for immunomodulators to be effective. Experiments were performed to study the presence of cell types in blood, liver, spleen, and bone marrow after administration of liposomal MTPPE and IFN–γ. These studies were supposed to give more insight in the development and recruitment of cells upon immunomodulation, and as such direct to further research on mechanisms behind immunomodulation.

(4) It was shown by others that colony stimulating activity levels in serum were increased in animals treated with immunomodulators. There are also indications that certain cytokines are involved in the cascade induced by immunomodulators which results in the observed host defense enhancement. However, little is know on the onset of cytokine production and cytokine network resulting from administration of immunomodulators. The presence of mRNA coding for important regulatory cytokines was studied in mice treated with liposomal MTPPE and IFN–γ. To detect mRNA a sensitive mRNA-PCR assay was developed.

(5) As already stated before, prophylactic treatment with immunomodulators to enhance antimicrobial host defence is believed to be of importance particularly in the immunocompromised host. A possible group recognized by us to receive this treatment are transplant recipients. The onset of immunodeficiency is known, and also the likelihood of acquiring a severe and life threatening infection. However, as one might expect, activation of host defense, certainly in a nonspecific way as achieved with immunomodulators, could at the same time result in accelerated graft rejection. This would mean that although patients might be protected against infection effectively by administration of immunomodulators, this treatment on the other hand induces rejection of the graft. We investigated skin graft rejection in mice which received liposomal MTPPE and IFN–γ. The aim of this study was to show that antimicrobial host defense activation is possible without causing transplant rejection.

(6) In vitro studies were performed to examine heterogeneity of Kupffer cells and splenic macrophages after priming with macrophage-activating cytokines and subsequent exposure to *K. pneumoniae*. Thereto a method was developed to isolate murine liver macrophages (Kupffer cells) and macrophages of spleen. In most of the in vitro studies described by us and other investigators peritoneal macrophages are used. From the

peritoneum large number of macrophages can be obtained quite easily. However, it is known that essential differences exist between tissue macrophages (i.e Kupffer cells or macrophages of the spleen) and macrophages of the peritoneal cavity. Moreover, it was shown that intravenous administration of the type of liposomes used in this study results in predominant localization of liposomes in macrophages of liver and spleen. Secondly, liver macrophages (Kupffer cells) and splenic macrophages are important in host defence towards septicemia.

(7) As Kupffer cells due to their localization and large number, play an important role in blood clearance and control of immunologic reactions, we studied the heterogeneity of these cells in more detail. TNF–α (produced by for instance macrophages) and IFN–γ (produced by T-cells and NK cells) are known to have strong activating effects on macrophages. In vitro experiments were performed on Kupffer cells after priming with TNF–α and/or IFN–γ followed by stimulation with *K. pneumoniae*.

CHAPTER TWO

Modulation of Nonspecific Antimicrobial Resistance of Mice to *Klebsiella pneumoniae* Septicemia by Liposome-encapsulated Muramyl Tripeptide Phosphatidylethanolamine and Interferon–γ Alone or Combined.

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ABSTRACT

Activation of the host defence system in a nonspecific way might provide tools to support failing antibiotic treatment in certain infectious diseases. The antimicrobial effect was investigated of liposome-encapsulated muramyl tripeptide phosphatidylethanolamine (MTPPE) and interferon (IFN)–γ and liposome-encapsulated MTPPE and IFN–γ on *Klebsiella pneumoniae* septicemia in mice. Prophylactic treatment of mice with five doses of liposomal MTPPE or IFN–γ increased survival from 0% to 65%. Administration of MTPPE and IFN–γ coencapsulated in liposomes resulted in 100% survival. In vitro, peritoneal macrophages by themselves were stimulated by these agents but were unable to kill *K. pneumoniae*. However, production of both oxygen and nitrogen intermediates increased when immunomodulators were added to macrophages. These results indicate that effective prophylactic treatment of septicemia due to *K. pneumoniae* with coencapsulated MTPPE and IFN–γ, is not solely due to activation of the resident macrophages.

INTRODUCTION

Malfunction of the host defence system renders patients more susceptible to (opportunistic) infections [1,2] and can make antibiotic treatment ineffective [1,3,4]. Stimulation of the host defence system, preferably in a nonspecific way so that not only certain bacterial but also viral infections and even malignancies can be treated, might provide effective treatment of infections in immunocompromised hosts.

Activation of cells of the mononuclear phagocyte system (MPS), in particular resident macrophages, by immunomodulating agents has proved beneficial in several infection models or in antitumor therapy [5-13]. Treatment of intracellular infections of the MPS seems obvious since most immunomodulating agents exert their effect mainly via these cells. Both muramyl tripeptide phosphatidylethanolamine (MTPPE), a derivative of muramyl dipeptide (MDP), and the cytokine interferon (IFN)–γ showed antibacterial activity in infections of the MPS [5,11,12,14-18].

Failure of antibiotic treatment is primarily seen in immunocompromised patients, who are more susceptible to infections and are also prone to infections for a relatively long period; therefore ,repeated administration with immunomodulating agents is needed [19,20]. However, most of these agents cause serious side effects when used repeatedly or in high dosages. Liposomal encapsulation of immunomodulating agents reduces toxicity by decreasing exposure of the body to the agent. Increased localization of the agent in MPS cells results in sequestering of the immunomodulator from sensitive sites [15,21-23]. In addition, infections of MPS cells and diseases controlled by these cells are treated more effectively with liposome-encapsulted immunomodulating agents than with solube agents. [11,24,25].

Liposome-encapsulated immunomodulators, unlike the solube form, are not excreted shortly after intravenous (iv) injection, and can be detected for a relatively long period (several days) in MPS cells [26]. Few reports, however, have examined the effect of nonspecific immunomodulation on infections outside the MPS. Such

infections, especially those with Gram-negative bacteria, are clinically important. Many immunocompromised patients develop septicemia, wich has a high case-fatality rate. It appears that a high blood clearance capacity of the MPS is vitally important for resistance to septicemia [27]. Activation of MPS cells might, therefore, benefit patients with infections outside the MPS.

Klebsiella pneumoniae is poorly taken up by MPS cells when not opsonized with specific antibodies [28]. It is therefore important to investigate if activation of cells of the MPS with immunomodulating agents can augment the phagocytic capacity, and by doing so, increase the uptake and killing of *K. pneumoniae* despite the absence of specific antibodies. Galland and colleagues [29-31] and Polk and colleagues [32,33] showed that *K. pneumoniae*-infected wounds can be treated to some extent with MDP. In addition, Hershman and colleagues [34-36] showed that treatment with IFN–γ increases resistance to intramuscular *K. pneumoniae* infections.

Previous studies by our group indicated that prophylactic treatment with a one dose of MTPPE (free or liposome-encapsulated) can increase resistance of mice to iv-induced *K. pneumoniae* infection [37]. However, in a model mimicing naturally acquired septicemia, the antimicrobial effect of treatment with liposome-encapsulated MTPPE was less pronounced [38]. In that model, bacteria were injected intraperitoneally, allowing intraperitoneal (ip) growth and dissemination of the microorganisms that in turn cause acute lethal septicemia. In the ip-inoculation septicemia model, bacteria continuously enter the blood, placing greater demand on the clearance capacity of the MPS than does one iv inoculation. We believe that this ip model is representative of septicemia acquired from local infection sites in patients.

In the present study, we investigated the effects of liposome-encapsulated MTPPE (LE-MTPPE), IFN–γ (LE-IFN–γ), and MTPPE plus IFN–γ (coencapsulation: LE-MTPPE/IFN–γ) on the survival of mice in a model of *K. pneumoniae*-induced lethal septicemia. The coencapsulation of MTPPE and IFN–γ allowed us to study potentiating effects between the two agents in vivo. The antimicrobial effect of LE-MTPPE and LE-IFN–γ was also studied in peritoneal macrophages infected with *K. pneumoniae*.

MATERIALS AND METHODS

Animals. Specified pathogen-free, 11- to 13- week-old female C57Bl/Ka mice were used (ITRI-TNO, Rijswijk, Netherlands).

Bacteria. Capsular serotype 2 *K. pneumoniae* (43816; American Type Culture Collection, Rockville, MD).

Reagents. LE-MTPPE and placebo liposomes (PL) were provided by Ciba Geigy (Basel, Switzerland). Recombinant rat IFN–γ was provided by Dr. P. van der Meide (ITRI-TNO), The Netherlands) in collaborative effort between the two laboratories. Liposomes containing MTPPE or IFN–γ and liposomes containing MTPPE plus IFN–γ were prepared as previously described [11]. Seven days before inoculation with bacteria, the mice received the first iv dose of LE-MTPPE; thereafter, doses were given every other day, with the last two at 24 and 12 h before bacterial inoculation. Fluorescein isothiocyanate (FITC) was obtained from Sigma (St. Louis, Missouri, USA).

Experimental infection caused by K. pneumoniae. Clearance of bacteria from the blood

was studied after iv inoculation of the mice with 10^4 cfu of *K. pneumoniae*. Blood samples were taken at different times after inoculation from the retroorbital plexus and diluted 2-fold in 3.8% sodium citrate (BDH, Poole, UK). Serial 10-fold dilutions were prepared and 0.2 mL volumes of each dilution were spread on tryptone soya agar (TSA) plates (Oxoid, Basingstoke, UK). A model of acquired septicemia was induced by ip inoculation of 10^3 cfu of *K. pneumoniae* into the mice. In this model multiplication of bacteria resulted in delayed but continuous appearance of bacteria in the blood of mice, eventually causing death from septicemia. Mice were monitored daily for survival until 28 days after bacterial inoculation. All mice that died during the experiment were examined for the presence of *K. pneumoniae* in the liver and blood. In the survival experiments, 20 mice were used per group. To study if animals that survived the infection were free of *K. pneumoniae*, mice were sacrificed and liver and spleen removed and homogenized in 20 mL of PBS for 30 s at 10,000 rpm in a homogenizer (VirTis, Gardiner, NY). The homogenate was mixed with an equal volume of double concentrated TSA and poured into plastic plates, which were then incubated overnight at 37°C .

Monolayers of peritoneal macrophages infected with K. pneumoniae. Monolayers of resident peritoneal macrophages from mice were cultured in a humidified atmosphere of 7.5% CO_2 at 37°C in chamber slides (Miles laboratories, Naperville, IL) in culture medium containing Dulbecco's MEM and 15% fetal bovine serum for 48 h before incubation with bacteria. At 12, 24, or 48 h before incubation with bacteria, the cells were exposed to LE-MTPPE, LE-IFN–γ or PL, or PBS. At zero time, bacteria were added in a ratio of 15 bacteria per macrophage. Uptake of *K. pneumoniae* by peritoneal macrophages was determined after 30 min by counting intracellular FITC-labeled bacteria [39], and killing was determined by preparing 10-fold serial dilutions of lysed macrophage cell suspension. The macrophages were lysed in ice-cold water with 0.01% bovine serum albumin, which did not effect viability of bacteria. Volumes of 0.2 mL were spread on TSA plates and incubated overnight at 37°C.

Fluorescent labeling of Liposomes. Liposomes were passively labeled with 0.03 mol % of rhodamine-phosphatidylethanolamine, a marker of lipid bilayers [40].

Quantification of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). H_2O_2 secretion was measured by modified horseradish peroxidase-catalyzed oxidation of phenol red adapted for microtiter plate [41]. Briefly, $5X10^5$ macrophages in 250 μL HBSS supplemented with 20% fetal calf serum (to which 250 μL of phenolred [0.025 mg/mL in HBSS] containing 19 units/mL horseradish peroxidase [Sigma] was added) were incubated with heat-killed bacteria at a ratio of 15 bacteria per macrophage for 1, 2 or 4 h. Oxidation of phenol red was stopped by the addition of 10% (vol/vol) of 1 *N* NaOH. A_{600} was determined and compared with a standard curve prepared at the same time using known concentrations of hydrogen peroxide.

Nitrite production was measured using the Griess reagents as reported by Stuehr et al [42]. Briefly, $5X10^5$ macrophages in 250 μL HBSS supplemented with 10% fetal calf serum were incubated with heat-killed bacteria at a ratio of 15 bacteria per macrophage for 1, 2 or 4 h. From every monolayer, 150 μL of supernatant was mixed with 150 μL of Griess reagents. A_{570} was determined and compared with a standard curve prepared at the same time using known concentrations of sodium nitrite.

Statistical analysis. Differences in survival curves among different groups of mice were evaluated by log rank test. The Kruskal-Wallis test was used to evaluate differences in the intracellular number of bacteria among monolayers of macrophages, for evaluation of killing of bacteria by macrophages, and for differences in ROI and RNI production.

RESULTS

Effect of repeated administration of liposomes. The liposomes used mainly target cells of the MPS and are phagocytosed by these cells. Overloading macrophages with liposomes might influence the uptake of bacteria from the blood and, by doing so, negatively influence the capacity of the macrophages. Mice were injected with 6.25 mg of PL, which correlates with the amount of lipid used in the other liposome-treated groups, or with PBS at 168, 120, 72, 24 and 12 h before iv inoculation with 10^4 cfu of *K. pneumoniae*. Administration of PL did not change the clearance capacity of the MPS cells compared with those of the controls: In both groups, 80% of the bacteria were cleared from the blood within the first 15 min and 90% at 60 min after inoculation (figure 1).

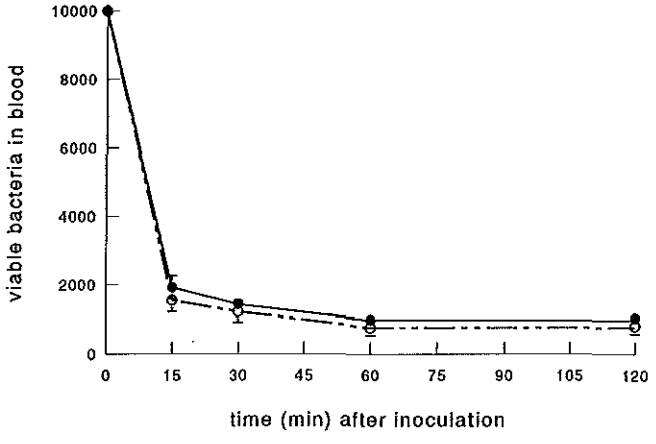

Figure 1. Clearance of *K. pneumoniae* from blood of mice treated with placebo liposomes (o) or PBS (•) 168, 120, 72, 24 and 12 h before bacterial challenge. Each point represents the mean ± SE of viable bacteria from 3 mice. In some cases, data points are bigger than error bars.

Effect of repeated administration of liposomal immunomodulators on survival. Sixty-five percent of the mice given LE-MTPPE (25 μg) or LE-IFN–γ (3125 units) at 168, 120, 72, 24 and 12 h before ip bacterial challenge survived 4 weeks after challenge (figure 2). However, all mice treated with the same dosages of co-encapsulated MTPPE and IFN–γ (LE-MTPPE/IFN–γ) were alive 4 weeks after challenge. All mice were examined for the presence of *K. pneumoniae*: The bacterium was found in the blood cultures and liver samples from all animals that died. Liver and spleen samples were cultured for *K. pneumoniae* in all surviving mice: None was detected 28 days after bacterial challenge. Repeated administration of 6.25 mg PL did affect on the survival: All mice died within 5 days of challenge. The survival of mice treated with PBS or PL did not differ (data not shown).

Effect of one dose of liposomal immunomodulators on survival. To investigate the combined effect of coencapsulated MTPPE and IFN–γ on the activation of the non-specific defence system in more detail, mice were given one dose of liposomal immunomodulators (figure 3). Survival from *K. pneumoniae* septicemia was increased from 0 to 30% when mice were treated prophylactically with one dose of LE-MTPPE (25 μg) 24 h before inoculation of bacteria, and 15% of mice survived when given one

dose of LE-IFN–g (3125 units). However, administration of one dose of LE-MTPPE/IFN–γ showed a potentiation of the antimicrobial effect, resulting in an increased survival of mice: 45% survived for 28 days after challenge.

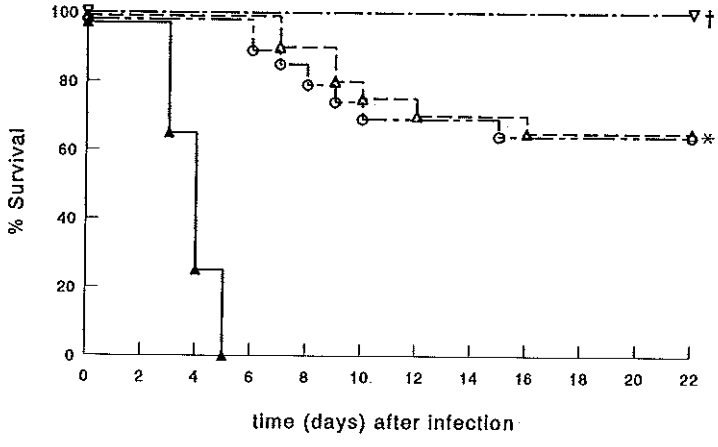

Figure 2. Survival of mice treated 168, 120, 72, 24 and 12 h before bacterial challenge, with 25-μg doses of LE-MTPPE (△) 3125-unit doses of LE-IFN–γ (○) or equal doses of LE-MTPPE/IFN–γ (▽). Controls were treated with placebo liposomes (PL;▲). Treatment groups had 20 mice each. Significance: $^{*}P < .001$ compared with PL-treated mice; $^{\dagger}P < .005$ compared with LE-MTPPE- or LE-IFN–γ treated mice.

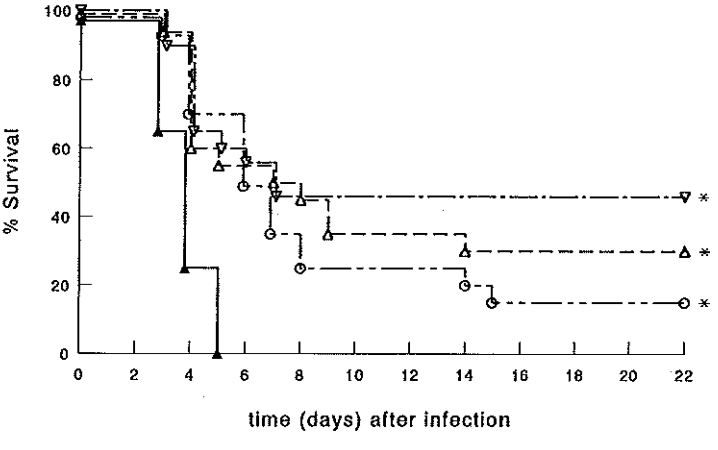

Figure 3. Survival of mice treated 24 h before bacterial challenge with single 25-μg dose of LE-MTPPE (△), 3125-unit dose of LE-IFN–γ (○), or equal dose of LE-MTPPE/IFN–γ (▽). Controls were given placebo liposomes (PL;▲). Treatment groups had 20 mice each. Significance: $^{*}P < .001$ compared with PL-treated mice.

Effect of liposomal immunomodulators on uptake and killing by macrophages in monolayer. K. pneumoniae is poorly phagocytosed by macrophages when not opsonized with specific antibodies [28]. In this experiment, the ability of macrophages in monolayer to take up and kill bacteria opsonized with nonimmune serum was studied. Exposure of monolayers of macrophages to LE-MTPPE, LE-IFN–γ or LE-(MTPPE/IFN–γ) for 24 h before addition of bacteria did not augment or diminish the uptake of *K. pneumoniae* by the macrophages (table 1). In addition, when exposure time was changed from 24 h to 12 or 48 h, no change in uptake of *K. pneumoniae* was

observed (data not shown). Figure 4 shows that intracellular *K. pneumoniae* increased 10-fold within 4 h after bacterial uptake in macrophage monolayers treated with PBS. Exposure of the monolayers to liposomal MTPPE, IFN–γ, or the combination of these agents, did not augment bactericidal activity. The number of intracellular bacteria in macrophages exposed to liposomal immunomodulators increased by the same order of magnitude as in macrophages exposed to PL or PBS.

Table 1. Effect of LE-MTPPE, LE-IFN-γ, and LE-(MTPPE/IFN-γ) on the uptake of Klebsiella pneumoniae by peritoneal macrophages in monolayer in a bacterium to cell ratio of 15 to 1

Treatment[a]	Number of bacteria per cell[b]	percentage of cells with intracellular bacteria
PBS	0	89.3 ± 2.1[c]
	1	6.6 ± 1.0
	2-5	2.9 ± 0.7
	>5	1.2 ± 0.3
Placebo Liposomes	0	91.1 ± 1.2
	1	5.9 ± 1.2
	2-5	2.8 ± 0.4
	>5	0.8 ± 0.3
LE-MTPPE	0	87.6 ± 1.9
	1	6.7 ± 1.2
	2-5	4.5 ± 0.9
	>5	1.2 ± 0.4
LE-IFN-γ	0	88.6 ± 2.8
	1	5.7 ± 0.6
	2-5	4.3 ± 0.5
	>5	1.3 ± 0.4
LE-(MTPPE/IFN-γ)	0	87.1 ± 2.6
	1	6.6 ± 1.1
	2-5	4.7 ± 1.0
	>5	1.6 ± 0.5

a Monolayers of peritoneal macrophages were exposed to 0.25 μg LE-MTPPE per mL, 31 units LE-IFN-γ per mL, 0.25 μg LE-MTPPE co-encapsulated with 31 units IFN-γ per mL, 62.5 μg placebo liposomes per mL, or PBS for a period of 24 h before infection with *K. pneumoniae*.

b On each slide 400 cells were microscopically screened on the uptake of intracellular fluorescein isothiocyanate-labeled bacteria.

c Percentages are the mean ± SEM for six experiments.

Uptake of liposomes by monolayers of macrophages. To ensure that liposome-encapsulated immunomodulating agents enter the macrophage, the uptake of rhodamine-labeled liposomes was examined. This labeling did not influence uptake by macrophages (data not shown). At 12 h after addition, rhodamine-labeled liposomes were detected in 38% of the macrophages. After 24 or 48 h, respectively, 35% and 30% of the macrophages contained fluorescent liposomes.

Detection of ROI and RNI produced by macrophage monolayers exposed to liposomal immunomodulators. Unstimulated macrophage monolayers produced very

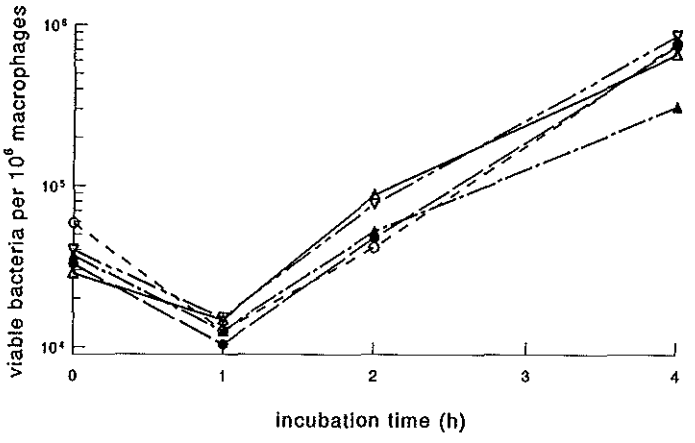

Figure 4. Numbers of viable intracellular bacteria in cultures of mouse peritoneal macrophage monolayers exposed to 0.25 μg/mL LE-MTPPE (▽), 3.1 units/mL LE-IFN–γ (▲), or equal concentrations of LE-MTPPE/IFN–γ (●), 62.5 μg/mL placebo liposomes (○) or PBS (△) for 24 h before infection with *K. pneumoniae*. Experiments were done 6 times.

small amounts of ROI and RNI after 48h of culture. However, incubation of the macrophages with heat-killed *K. pneumoniae* resulted in 2-fold increase in NO_2^- and H_2O_2 production (table 2). The production of NO_2^- by macrophages stimulated with heat-killed *K. pneumoniae* was augmented by preexposure for 24 h with LE-MTPPE and LE-IFN–γ after 2 h of incubation with bacteria. LE-MTPPE/IFN–γ even further stimulated NO_2^- production ($P < .01$ compared with LE-MTPPE- and LE-IFN–γ-treated macrophages). However, at 4 h after incubation of the macrophages with bacteria, production of NO_2^- diminished. NO_2^- production by LE-IFN–γ-, LE-MTPPE-, and LE-MTPPE/IFN–γ-stimulated macrophages was enhanced compared with that in PL treated cells.

Table 2. Effect of LE-MTPPE, LE-IFN-γ and LE-(MTPPE/IFN-γ) on the production of nitrogen oxide and hydrogen peroxide by peritoneal macrophages in monolayer incubated with heat killed *K. pneumoniae*.

	Intermediate production by h after bacterial inoculation					
Treatment	NO_2^-			H_2O_2		
(per mL of Macrophages)*	1	2	4	1	2	4
PBS	100 (9.1)	100 (9.7)	100 (8.3)	100 (3.17)	100 (2.45)	100 (2.99)
Placebo Liposomes	99 (9.0)	95 (9.2)	97 (8.1)	126 (3.99)	226 (5.54)	228 (6.82)
LE-MTPPE	112 (10.2)	132 (12.8)†	112 (9.3)	137 (4.34)	230 (5.64)	226 (6.76)
LE-IFN-γ	97 (8.8)	203 (19.7)†	123 (10.2)†	179 (5.67)	309 (7.57)†	251 (7.49)
LE-(MTPPE/IFN-γ)	120 (10.9)	319 (30.9)†§	144 (11.9)†§	226 (7.16)	312 (7.64)†	268 (7.97)§

NOTE. Data are % compared with PBS-treated controls (nmol/10^6 macrophages). Bacteria to cell ratio was 15 to 1.
* Exposure for 24 h before infection with *K. pneumoniae*.
Significance: †$P < .01$ compared with PL-treated group; $P < .001$ compared with PL-treated group, $P < .01$ compared to LE-MTPPE- or LE-IFN–γ-treated groups; §$P < .05$ compared with PL-treated group.

Macrophages stimulated with heat-killed *K. pneumoniae* and preexposed to PL had significantly increased production of H_2O_2 compared with PBS-treated controls ($P < .01$) at 2 and 4 h after addition of the bacteria. Although the production of H_2O_2 increased with administration of LE-MTPPE and LE-IFN–γ at all time points, only exposure to LE-IFN–γ and LE-MTPPE/IFN–γ increased the production significantly at 2 h and at 2 and 4 h, respectively, compared with the PL treated controls.

DISCUSSION

The use of liposomes as carriers of chemotherapeutic or antimicrobial agents has been previously examined [8,23,25,26,43,44]. Administration of liposome-encapsulated agents has many advantages: reduction of toxicity induced by potentially toxic agents, prolongation of half-life, and targeting of agents to specific sites. In the present study, the type of liposomes we used, negatively charged multilamellar vesicles, are primarily taken up by the cells of the MPS (reviewed in [23,26]).

It was shown that when macrophages infected with *Listeria monocytogenes* in vitro were simultaneously exposed to MTPPE and IFN–γ, a synergistic antibacterial effect resulted in intracellular killing of the bacteria [11]. The value of this synergistic effect for treatment of infections is questionable since in vivo the simultaneous exposure of macrophages to additional immunomodulators after iv administration of the agents is expected to be minimal. However, a potentiating effect in vivo could be realized by administration of immunomodulators encapsulated in the same liposome.

Although MTPPE and IFN–γ increase host resistance towards malignancies and infections of the MPS [6,8,11,15,16,18,45,46], only a few reports demonstrate the potency of these agents in infections outside the MPS. Hershmann and colleagues [34-36] showed that a *K. pneumoniae* wound infection in mice could be succesfully treated to some extent with IFN–γ; however, only low end point survival rates were obtained, and the experimental infection was not universally lethal. In addition, prophylactic treatment of mice with MDP increased host resistance to *K. pneumoniae* wound infection; however, the infections were not always lethal [29-33]. Other models also showed the ability of MDP to augment host resistance to *K. pneumoniae* [47-49].

In the present paper, we used a mouse model of *K. pneumoniae* septicemia: Infection with *K. pneumoniae* was induced intraperitoneally, which allowed the bacteria to grow and disseminate to the blood, resulting in a model of acquired septicemia, with a 100% fatality rate within 5 days. This model is of clinical relevance because as a result of locally insufficient host defense mechanisms, immunodeficient patients often develop septicemia from a focal infection that disseminates into the blood [4,33].

Maximal blood clearance capacity of the MPS is important for immunocompromised patients. Treatment with immunomodulating agents may benefit such patients by enhancing the capacity of MPS cells to clear bacteria from the blood. Since immunodeficient patients are vulnerable to opportunistic infections for prolonged periods, repeated administration of immunomodulators is anticipated [19,20]. However, MTPPE as well as IFN–γ induce serious toxic side effects, so dosages must be limited [21,22,46,50]. To lessen toxicity we encapsulated MTPPE and IFN–γ in liposomes, thus reducing exposure of the agent to all body tissues. In addition, the efficacy of the agents is increased by liposomal targeting to MPS cells, thus allowing lower dosages to be effective [11,23-25].

Repeated administration of liposome-encapsulated immunomodulators results in exposure of MPS cells to large amounts of liposomes. Liposome saturation of MPS cells might occur and negatively influence the clearance of bacteria from the blood. Therefore it is important to use dosages that will not interfere with the phagocytic capacity of the MPS cells.

In a sensitive assay, we showed that the clearance capacity of the MPS was not diminished when a 6.25-mg dose of lipid was administered repeatedly. Prophylactic treatment of mice with 5-fold doses of liposomes containing 25 μg of MTPPE (LE-MTPPE) resulted in the survival of 65% of the animals at 28 days after bacterial challenge; this data agree with previous results in our laboratory [38]. In addition,

treatment with 5-fold dose of LE-IFN–γ increased survival from 0% to 65%. Moreover, all mice treated with LE-MTPPE/IFN–γ survived. In this model of *K. pneumoniae* septicemia, these results indicate a potentiating effect between MTPPE and IFN–γ when encapsulated into the same liposome. We and other investigators have previously shown in vitro and in vivo synergy between these two agents in intracellular infections with *L. monocytogenes* and in tumor models [11,45,51-53].

K. pneumoniae is effectively phagocytosed by macrophages or granulocytes only in the presence of specific antibodies [28]. We investigated whether activation of the phagocytic activity of MPS cells with immunomodulating agents might enable or increase phagocytosis of *K. pneumoniae* even in the absence of specific antibodies. We have shown previously that depletion of tissue macrophages completely eliminates any antimicrobial effect of LE-MTPPE against *K. pneumoniae* septicemia [38]. Those results indicate that activation of tissue macrophages by LE-MTPPE is of major importance in the induced increase in host resistance, although recruitment of leukocytes from the bone marrow to the blood due to LE-MTPPE administration also contributes significantly to augmentation of the host defence system [38].

To test this, we examined the effect of LE-MTPPE and LE-IFN–γ alone or together (LE-MTPPE/IFN–γ) on the phagocytic capacity of macrophages in culture. Hockertz et al. [15] demonstrated an increase in killing of *Leishmania* species by macrophages treated with LE-MTPPE, LE-IFN–γ or a combination of these agents. Melissen et al. [11] demonstrated a synergistic effect between MTPPE and IFN–γ on the ability of macrophages to kill *L. monocytogenes*.

In these models, effects were shown with intracellular microorganisms. We, on the other hand, were not able to show any effect on the uptake or killing of *K. pneumoniae* by monolayers of peritoneal macrophages exposed to LE-MTPPE, LE-IFN–γ or LE-MTPPE/IFN–γ. The discrepancy may be explained by the type of microorganism used. *L. monocytogenes* is easily taken up by macrophages and grows intracellularly without activating macrophages. However, as soon as the macrophages are activated by MTPPE or IFN–γ, the bacteria are digested. *K. pneumoniae,* on the other hand, is well-equipped against macrophages by preventing phagocytosis as a result of a polysaccharide capsule. Even when phagocytosed, this bacterium is still hard to kill, as was shown in this study and by others [54].

Previous in vitro experiments demonstrate that exposure of macrophages to IFN–γ does not result in enhanced uptake or killing of *Pseudomonas aeruginosa* and *Staphylococcus aureus* [55]. Moreover, exposure of macrophages to IFN–γ results in suppression of their antimicrobial activity. Therefore, macrophage must be fully activated to result in killing of the ingested bacteria.

The detection of ROI and RNI demonstrates that peritoneal macrophages in monolayer exposed to heat-killed bacteria are viable and capable of producing large amounts of toxic intermediates. Production of the toxic intermediates was shown to be augmented by LE-MTPPE, LE-IFN–γ or LE-MTPPE/IFN–γ. We previously showed that activation of macrophages with MTPPE plus IFN–γ results in killing of *L. monocytogenes* [11]. However, our current results suggest that activation of the macrophage alone is not enough to enhance uptake or intracellular killing of *K. pneumoniae*. Other cells are probably necessary, not only for optimal activation of macrophages via cytokines or by communication via cell-to-cell contact, but also through the antimicrobial activity of other effector cells.

We demonstrated in our in vivo model that multiple treatments with liposome-encapsulated immunomodulators is more effective then a single injection. These results indicate that the MPS cells do not become refractory to stimulation and new cells are

activated with each subsequent dose. However, the treatment procedure that we used is relatively short compared with the clinical setting in which patients are treated for weeks or months. Studies in which liposomal MTPPE was administered biweekly for 9 months showed that monocytes do not became refractory to treatment [56,57]; however, this must be investigated in more detail our model to see whether prolonged treatment will remain effective.

REFERENCES

1. Soave R, Sepkowitz KA. The immunologic compromised host. In: Reese RE, Betts RF, ed. A practical approach to infectious diseases. London, Litlle, Brown and Company 1991:566-607.
2. Van der Meer JWM. Defects in host defence mechanisms. In: Rubin RH, Young LS. Clinical approach to infection in the immunocompromised host. New York: Plenum medical book company, 1988:41-73.
3. Bone R. Gram-negative sepsis: a dilemma of modern medicine. Clin Microbiol Reviews 1993;6(1):57-68.
4. Umsawasdi T, Middleman EA, Luna M, and Bodey G. Klebsiella bacteremia in cancer patients. Am J Med Sci 1973;265:474-82.
5. Badaro R, Johnson Jr WD. The role of interferon-γ in the treatment of visceral and diffuse cutaneous leishmaniasis. J Infect Dis 1993;167(Suppl 1):S13-7.
6. Czarniecki CW, Sonnenfeld G. Interferon-gamma and resistance to bacterial infections. APMIS 1993;101:1-17.
7. Fraser-Smith EB, Eppstein DE, Larsen MA, Matthews TR. Protective effect of a muramyl dipeptide analog encapsulated in or mixed with liposomes against candida albicans infection. Infect Immun 1983;39(1):172-8.
8. Frost H. MTP-PE in liposomes as a biological response modifier in the treatment of cancer: Current status. Biotherapy 1992;4(3):199-204.
9. Koff WC, Showalter SD, Hampar B, Fidler IJ. Protection of mice against fatal herpes simplex type 2 infection by liposomes containing muramyl tripeptide. Science 1985;228:495-7.
10. Kurtz RS, Young KM, Czuprynski CJ. Seperate and combined effects of recombinant interleukin-1α and gamma interferon on antibacterial resistance. Infect Immun 1989;57(2):553-8.
11. Melissen PMB, Van Vianen W, Bidjai O, Van Marion M, Bakker-Woudenberg IAJM. Free versus liposome-encapsulated muramyl tripeptide phosphatidylethanolamide (MTPPE) and interferon-γ (IFN-γ) in experimental infetion with *Listeria monocytogenes*. Biotherapy 1993;6:113-24.
12. O'Reilly T, Zak O. Enhancement of the effectiveness of antimicrobial therapy by muramyl peptide immunomodulators. Clin Infect Dis 1992;14:1100-9.
13. Vogels MTE, Van der Meer JWM. Use of immune modulators in nonspecific therapy of bacterial infections. Antimicrob Agents Chemother 1992;36(1):1-5.
14. Fevrier M, Birrien JL, Leclerc C, Chedid L, Liacopoulos P. The macrophage, target cell of the synthetic adjuvant muramyl dipeptide. Eur J Immunol 1978;8:558-62.
15. Hockertz S, Franke G, Paulini I, Lohmann-Matthes M-L. Immunotherapy of murine visceral leishmaniasis with murine recombinant interferon-γ and MTP-PE encapsulated in liposomes. J Interferon Res 1991;11(3):177-85.
16. Matsumura H, Onozuka K, Terada Y, Nakano Y, Nakano M. Effect of murine recombinant interferon-γ in the protection of mice against *Salmonella*. Int J Immunopharmacol 1990;12:49-56.
17. Melissen PMB, Van Vianen W, Hofkens MAM, Van Opstal A, Bakker-Woudenberg IAJM. Effect of muramyl tripeptide phosphatidylethanolamide (MTPPE) and interferon-gamma (IFN-γ) on *Listeria monocytogenes* infection in genetically resistant or susceptible mice. Immunol Infect Dis 1991;3:89-96.
18. Reed SG. In vivo administration of recombinant IFN-γ induces macrophage activation, and prevents acute disease, immune suppression, and death in experimental *Trypanosoma Cruzi* infections. J Immunol 1988;140(12):4342-7.
19. the EORTC International Antimicrobial Therapy Coorperative Group. Ceftazidime combined with a short or long course of amikacin for emperical therapy of gram-negative bacteremia in cancer patients with granulocytopenia. New Engl J Med 1987;317:1692-8.
20. Klastersky J. Empiric treatment of infections in neutropenic patients with cancer. Rev Infect Dis 1983;5:S21-31.

21. Fidler IJ, Brown NO, Hart JR. Species variability for toxicity of free and liposome-encapsulated muramyl peptides administred intravenously. J Biol Response Modif 1985;4:298-309.
22. Schumann GP, Van Hoogevest P, Fankhauser P, et al. Comparison of free and liposomal MTPPE: Pharmacological, toxicological and pharmacokinetic aspects. In: Lopez-Berestein G, Fidler IJ. Liposomes in the therapy of infectious disease and cancer. UCLA, Alan R Liss Inc. New York: Symposium on Molecular and Cellular Biology, New Series, 1989;89:191-203.
23. Lasic D. Liposomes. American Scientist 1992;80(1):20-31.
24. Daemen T, Veninga A, Roerdink FH, Scherphof GL. In vitro Activation of rat liver macrophages to tumoricidal activity by free or liposome-encapsulated muramyl dipeptide. Cancer Res 1986;46:4330-5.
25. Nayar R, Fidler IJ. The systemic activation of macrophages by liposomes containing immunomodulators. Springer Semin Immunopathol 1985;8:413-28.
26. Pak, CC, Fidler IJ. Liposomal delivery of biological response modifiers to macrophages. Biotherapy 1991;3:55-64.
27. Melissen PMB, Van Vianen W, Bakker-Woudenberg IAJM. Roles of peripheral leukocytes and tissue macrophages in antimicrobial resistance induced by free or liposome-encapsulated muramyl tripeptide phosphatidylethanolamide. Infect immun 1992;60(11):2891-7.
28. Undeutsch C, and Brunner H. Influence of antibodies on the phagocytosis of *Klebsiella pneumoniae* by alveolar macrophages. Zbl Bakt Hyg, I Abt Orig A 1981;249:43-52.
29. Galland RB, Trachtenberg LS, Rynerson N, Polk Jr HC. Nonspecific enhancement of resistance to local bacterial infection in starved mice. Arch Surg 1983;118:161-4.
30. Galland RB, Heine KJ, Polk HC. Nonspecific stimulation of host defenses against bacterial challenge in immunosuppressed mice. Arch Surg 1983;118:333-7.
31. Galland RB, Polk HC. Non-specific stimulation of host defenses against a bacterial challenge in malnourished hosts. Br J Surg 1982;69:665-8.
32. Polk HC, Galland RB, Ausobsky FR. Nonspecific enhancement of resistance to bacterial infection evidence of an effect supplemental to antibiotics. Ann Surg 1982;196:436-41.
33. Polk HC, Lamont PM, Galland RB. Containment as a mechanism of non-specific enhancement of defences against bacterial infection. Infect Immun 1990;158:1807-11.
34. Hershman MJ, Sonnenfeld G, Mays BW, Flemming F, Trachtenberg LS, Polk HC. Effects of interferon–γ treatment on surgically stimulated wound infection in mice. Micribial Pathogen 1988;4:165-8.
35. Hershman MJ, Polk HC, Pietsch JD, Kuftinec D, Sonnenfeld G. Modulation of *Klebsiella pneumoniae* infection of mice by interferon–γ. Clin Exp Immunol 1988;72:406-9.
36. Hershman MJ, Polk HC, Pietsch JD, Shields E, Wellhausen SR, Sonnenfeld G. Modulation of infection by gamma interferon treatment following trauma. Infect Immun 1988;56(9):2412-16.
37. Melissen PMB, Van Vianen W, Rijsbergen Y, Bakker-Woudenberg IAJM. Free versus liposome-encapsulated muramyl tripeptide phosphatidylethanolamine in treatment of experimental *Klebsiella pneumoniae* infection. Infect Immun 1991;60(1):95-101.
38. Melissen PMB, Van Vianen W, Bakker-Woudenberg IAJM. Treatment of *Klebsiella pneumoniae* septicemia in normal and leukopenic mice by liposome-encapsulated muramyl tripeptide phophatidylethanolamide. Antimicrob Agents Chemother 1994;38(1):147-150.
39. De Graaff-Miltenburg LAM, Van Vliet KE, Ten Hagen TLM, Verhoef J, Van Strijp JAG. The role of HSV-induced Fc- and C3b(i)-receptors in bacterial adherence. J Med Microbiol 1994;40:48-54.
40. Verkade HJ, Zaal KJM, Derksen JTP, Vonk RJ, Hoekstra D, Kuipers F, and Scherphof GL. Processing of the phopholipid analogue phophatidyl(N-sulphorhodamine B sulphonyl)ethanolamine by rat in vitro and in vivo. Biochem J 1992;284:259-265.
41. Pick E, Mizel D. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. J Immunol Methods 1981;46:211-26.
42. Stuehr DJ, Nathan CF. Nitric oxide a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J Exp Med 1989;169:1543-55.
43. Bakker-Woudenberg IAJM, Lokerse AF, Melissen PMB, Van Vianen W, Van Etten EWM. Liposomes as carriers of antimicrobial agents or immunomodulatory agents in the treatment of infections. Eur J Clin Microbiol Infect Dis Suppl 1993;1:61-7.
44. Coune A. Liposomes as drug delivery system in the treatment of infectious diseases potential applications and clinical experience. Infection 1988;16:141-6.
45. Fidler IJ, Fan D, Ichinose Y. Potent in situ activation of murine lung macrophages and therapy of melanoma metastases by systemic administration of liposomes containing muramyltripeptide phosphatidylethanolamine and interferon-gamma. Invas Metastas 1989;9:75-88.
46. Billiau A, Dijkmans R. Interferon–γ: Mechanism of action and therapeutic potential. Biochem

Pharmacol 1990;40(7):1433-1439.
47. Chedid L, Parant M, Parant F, Lefrancier P, Choay J, Lederer E. Enhancement of nonspecific immunity to *Klebsiella pneumoniae* infection by a synthetic immunoadjuvant (N-Acetylmuramyl-L-alanyl-D-isoglutamine) and several analogs. Proc Natl Acad Sci USA 1977;74(5):2089-93.
48. Izbicki JR, Readler C, Anke A, et al. Beneficial effect of lipsome-encapsulated muramyl-tripeptide in experimental septicemia in a porcine model. Infect Immun 1991;59:126-30.
49. Parant M, Parant F, Chedid L. Enhancement of the neonate's nonspecific immunity to *Klebsiella* infection by muramyl dipeptide, a synthetic immunoadjuvant. Proc Natl Acad Sci USA 1978;75(7):3395-9.
50. Braun DG, Dukor P, Lukas B, et al. MTPPE, A synthetic lipophilic muramyltripeptide: Biological and toxocological properties. In: Berlin A. Immunotoxocology Dordrecht, the Netherlands: Nijhof Publisher, 1987:219-33.
51. Nagao S, Sato K, Osada Y. Augmentation by priming with interferon--γ of the binding of a muramyl dipeptide derivative to macrophages resulting in synergistic macrophage activation. Jpn J Cancer Res 1987;78:80-6.
52. Saiki I, Sone S, Fogler WE, Kleinerman ES, Lopez-Berestein G, Fidler IJ. Synergism between human recombinant γ-interferon and muramyl dipeptide encapsulated in liposomes for activation of antitumor properties in human blood monocytes. Cancer Res 1985;45:6188-93.
53. Saiki I, Fidler IJ. Synergistic Activation by Recombinant Mouse Interferon--γ and Muramyl Dipeptide of Tumoricidal Properties in Mouse Macrophages. J Immunol 1985;135(1):684-8.
54. Cuffini AM, Tullio V, Fazari S, Allocco A, Carlone NA. Pefloxacin and immunity: cellular uptake, potentiation of macrophage phagocytosis and intracellular bioactivity for *Klebsiella pneumoniae*. Int J Tiss React 1992;XIV(3):131-9
55 Speert DP, Thorson L. Suppression by human recombinant gamma interferon of in vitro macrophage nonopsonic and opsonic phagocytosis and killing. Infect Immun 1991;59(6):1893-8.
56. Kleinerman ES, Murray JL, Snyder JS, Cunningham JE, Fidler IJ. Activation of tumoricidal properties in monocytes from cancer patients following intravenous administration of liposomes containing muramyl tripeptide phophatidylethanolamine. Cancer Res 1989;49:4665-70.
57. Murray JL, Kleinerman ES , Cunningham JE, et al. Phase I trial of liposomal muramyl tripeptide phosphatidylethanolamine in cancer patients. J Clin Oncol 1989;7:1915-25.

CHAPTER THREE

Involvement of T cells in Enhanced Resistance to *Klebsiella pneumoniae* Septicemia of Mice Treated with Liposome-encapsulated MTPPE and IFN–γ.

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ABSTRACT

We have previously shown that prophylactic administration of the liposome-encapsulated immunomodulating agents MTPPE and IFN–γ results in strongly increased survival of mice from a normally lethal septicemia with *Klebsiella pneumoniae*. It was anticipated that the treatment acts on macrophages and non-specifically augments host resistance to various infections. In the present study we provide evidence for a key role for T-cells in host defence potentiation by the liposomal immunomodulators towards *Klebsiella pneumoniae* septicemia. It is shown that both CD4 and CD8 positive cells are important in immunomodulation, most likely due to production of IFN–γ. Depletion of circulating IFN–γ resulted in strong reduction of the antimicrobial host defence activation. Administration of IL-10 resulted in decreased antimicrobial host defence activation by liposomal immunomodulators. Moreover, administration of liposomal immunomodulators was shown to induce predominantly Th1 cell populations in the spleen. These findings indicate that immunomodulation with liposomal MTPPE and IFN–γ favors Th1 and NK cells activation.

INTRODUCTION

In several models of infection or tumor therapy non-specific stimulation of the host defence system has been convincingly shown to be effective. We have previously demonstrated that prophylactic treatment of mice to a lethal *Klebsiella pneumoniae* septicemia with liposome-encapsulated muramyl tripeptide phosphatidylethanolamine (LE-MTPPE) or interferon–γ (LE-IFN–γ), or both immunomodulators co-encapsulated in liposomes (LE-MTPPE/IFN–γ) could substantially augment survival [1]. As the liposomes used in these studies are taken up primarily by cells of the mononuclear phagocyte system (MPS), it is suggested that these cells are the primary target for the liposomal immunomodulators [2]. Macrophages were shown to exhibit intracellular receptors for both immunomodulators [3-5], and we demonstrated that these cells are of major importance in the observed host defence potentiation with LE-MTPPE in vivo [6].

Stimulation of purified macrophages in vitro with LE-MTPPE, LE-IFN–γ, or LE-MTPPE/IFN–γ resulted in enhanced production of oxygen and nitrogen metabolites, but not in increased phagocytic activity in an infection with *K. pneumoniae* [1]. These data suggest that activation of MPS cells is not sufficient to obtain effective killing of this microorganism. It appeared that these cells lacked important co-stimulating signals in vitro. Therefore, it is hypothesized that T-cells are involved in activation of macrophage or MPS as a whole. This is supported by studies with mice treated with cyclosporin A before treatment with liposome-encapsulated immunomodulators. Cyclosporin A preteratment resulted in a dramatic reduction of antimicrobial host defence potentiation towards *K. pneumoniae* infection induced by immunomodulators [7]: the 100% survival rate effected by LE-MTPPE/IFN–γ was reduced to 0%.

In the present paper the contribution of T-cells and their cytokines, to the efficacy of immunomodulation with LE-MTPPE, LE-IFN–γ or LE-MTPPE/IFN–γ to *K. pneumoniae*

septicemia was studied in vivo. To this end, mice were depleted of T-cells or T-cell subsets, or the production of specific cytokines (e.g. IFN–γ and TNF–α) was modulated by treatment with neutralizing antibodies. Here we demonstrate, for the first time, an important role for T-cells, and in particular Th1-cells in non-specific immunomodulation.

MATERIALS AND METHODS

Animals. Specific pathogen-free, female C57Bl/Ka mice, 11 to 13 weeks of age, were used (ITRI-TNO, Rijswijk, The Netherlands).

Bacteria. K. pneumoniae, capsular serotype 2 (ATCC 43816) were grown for 16 h at 37°C in Todd-Hewitt broth (Oxoid Ltd, Basingstoke, UK). The bacteria were preserved on ice and washed three times in PBS directly before use.

Reagents. Liposome-encapsulated muramyl tripeptide phosphatidyl ethanolamine (LE-MTPPE) was kindly provided by Ciba-Geigy (Basel, Switzerland). Placebo liposomes (PL) were prepared from a dry lyophilisate composed of phosphatidylcholine and phosphatidylserine in a molar ratio of 7:3 as previously described [1]. Recombinant murine IFN–γ was derived from supernatant fluid of a CHO cell line that carries and expresses an amplified murine IFN–γ cDNA [8]. Murine IFN–γ was used at a concentration of $2.5X10^5$ U/ml. IFN–γ was purified by affinity chromatography on an anti-rat IFN–γ mAb (DB-1) to a specific activity of $6X10^6$ U/mg. Liposomes containing MTPPE, IFN–γ and MTPPE plus IFN–γ were prepared as described previously [1].

Treatment of mice with immunomodulators. Mice were injected iv with LE-MTPPE, LE-IFN–γ or LE-MTPPE/IFN–γ as previously described [1]. Briefly, mice were injected five times every other day with 25 μg LE-MTPPE, or 7500 U LE-IFN–γ, or 25 μg MTPPE and 7500 U IFN–γ co-encapsulated in 6.25 mg lipid. Twelve hours after the last dose mice were infected i.p. with *K. pneumoniae* or mock-infected with PBS.

Depletion of T- cells and T-cell subsets. Mice were depleted of T-cells by a single ip injection of 100 μg 17A2, a mouse CD3-specific rat IgG2b mAb [9], at 24 h before the first dose with immunomodulator. Depletion of CD4- or CD8-positive cells was obtained by three ip injections of 100 μg YTS-191 or YTS-169, a CD4- and CD8-specific rat IgG2b mAb respectively [10]. These antibodies were administered at 72, 48 and 24 h before first dose with immunomodulator. Depletion of T-cells was evaluated in the blood and spleen by flow cytometry after staining with anti-CD3 mAb KT3 [11]. Controls for injection of IgG2b mAb were injected with equal amounts of a rat IgG2b anti-ß-galactosidase mAb (PH2-49)(a kind gift of Prof. Dr. W. Van Ewijk, Dept of Immunology, Erasmus University, Rotterdam, the Netherlands).

Depletion of endogenous IFN–γ. Endogenous IFN–γ was depleted by two ip injections of 100 μg F3, a mouse IFN–γ-specific rat IgG2a mAb [12], at 48 h before the first dose and 12 h after the last dose with immunomodulator. This dose and treatment scheme was shown to neutralize all bioactive IFN–γ in vivo. F3 was given as plain ascites fluid (in vitro neutralizing titer of 10^5 against 30 U/ml of murine IFN–γ, rat Ig content 3.5 mg/ml). Side-effects induced by injections with IgG2a antibody F3 were evaluated using a control GL117 rat IgG2a anti-ß-galactosidase mAb (obtained by the courtesy of Dr J. Abrams, DNAX Research Inst., Palo Alto, CA) in control ascites fluid at equal dose.

Administration of IL-10. Mice were injected ip 24 h before the first dose of immunomodulator with $2X10^6$ murine IL-10 cDNA transfected J558 cells encapsulated in alginate as previously described, resulting in elevated cytokine levels in vivo [13]. This cell dose has been widely shown for several cytokine transfected cell lines to be effective in vivo. Side-effects of alginate-IL-10 injections were evaluated using mock-transfected J558 cells encapsulated in alginate.

These cell lines were the kind gift of Dr K. Moore (DNAX Research Inst., Palo Alto, CA).

Depletion of TNF–α. Endogenous TNF–α was depleted by a single ip injection, at 48 h before first dose of immunomodulator, of an optimally titrated dose of 300 μg TNF–α -specific rabbit IgG isolated from hyperimmune serum. Controls were injected with the same amount of rabbit IgG isolated from normal serum.

Experimental infection with K. pneumoniae. Acquired septicemia was induced by ip inoculation of 10^3 cfu of *K. pneumoniae* in mice as described previously [1]. In this model, multiplication of bacteria resulted in delayed but continuous appearance of bacteria in the blood, eventually leading to septicemia and resulting in death. Mice were monitored daily for survival until 28 days after bacterial inoculation. All mice that died during the experiment were examined for the presence of *K. pneumoniae* in liver and blood. Per group 20 mice were used.

Evaluation of MHC-II (Ia) antigen expression of spleen macrophages by flow cytometry. At 12 h and 4 days after end of treatment spleen were excised, treated with collagenase and minced into cell suspensions as described elsewhere [14]. Cells were stained with mAb M5/114 [15] directed against Ia antigen and expression was measured by flow cytometry on a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA). During analysis lymphocytes were gated out. Expression was calculated as molecules equivalent to soluble FITC according to Leenen et al [15]. Three mice were used per treatment.

Examination of activity of Th1 and Th2 cells in spleen after immunomodulation. T-cell pulse-chase experiments were performed as described previously [17]. Briefly, to examine the effect of immunomodulation on the activity of Th1 and Th2 cells, spleens were excised from treated mice at 12 h and 4 days after end of treatment. The spleens were minced into a cell suspension, washed and resuspended at 200.000 cells per well and cultured on anti-CD3 mAb-coated 96 well culture plates. Cells were stimulated with medium or IL-2 (200 U/ml) alone (controls), or with IL-2 (200 U/ml) and IFN–γ (400 U/ml) accompanied by anti-IL-4 mAb (11B11 [17], 40 μg/ml)(Th1 pulse), or with IL-2 (200 U/ml) and IL-4 (140 ng/ml) accompanied by anti-IFN–γ mAb (XMG1.2 [18], 20 μg/ml)(Th2 pulse). After 4 days of culture at 37°C cells were washed two times and transferred to 96 well plates freshly coated with anti-CD3 mAb and chased with IL-2 (100 U/ml) to induce production of cytokines. After 4 days of culture at 37°C supernatants were harvested and IFN–γ and IL-4 levels were measured by sandwich ELISA as described previously [17,18].

Statistical analysis. Differences in survival curves among groups of mice were evaluated by log rank test. P-values below 0.05 were considered significant.

RESULTS

Effect of depletion of T-cells on augmentation of antimicrobial host defence by liposomal immunomodulators. It was shown before that treatment of immunocompetent mice with LE-MTPPE or LE-IFN–γ resulted in a dramatic increase in host defence; 65% of the mice survived whereas all control mice, treated with PL, died within 5 days after inoculation. Treatment with immunomodulators combined by co-encapsulation enhanced survival to 100%[1]. Control experiments which were performed during the present study also demonstrated that LE-MTPPE treatment (n = 9) or LE-IFN–γ (n = 9) treatment resulted in 65% and 60% survival respectively, 21 days after infection (data not shown). Confirmatory experiments on treatment with LE-MTPPE/IFN–γ performed in this study also demonstrated a 100% survival of the mice (shown in Fig. 1). Depletion of T-cells with anti-CD3 mAb 17A2 dramatically reduced the potency of the immunomodulators (Fig. 1). In

LE-MTPPE/IFN–γ treated mice survival decreased from 100% in immunocompetent mice to 15% in T-cell depleted mice ($P < .001$ compared with the immunocompetent controls). Survival of LE-MTPPE treated mice or LE-IFN–γ treated mice was reduced from 65% in immunocompetent mice to 15 or 5% respectively, in T-cell depleted mice. Because LE-MTPPE/IFN–γ treatment is superior to treatment with single immunomodulators, in the remainder of this study emphasis is put on the combination.

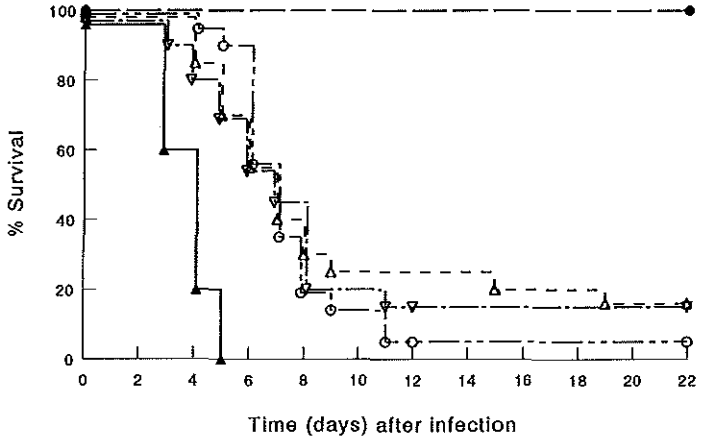

Figure 1. Survival of T-cell depleted mice infected by i.p. inoculation of 10^3 *Klebsiella pneumoniae*. T-cells were depleted by injection of CD3 specific IgG2b mAb. Mice were treated before infection with LE-MTPPE/IFN–γ (▽), LE-MTPPE (△), LE-IFN–γ (○) or PL (▲). Control mice were injected with rat IgG2b anti-ß-galactosidase mAb and treated with LE-MTPPE/IFN–γ (●). All groups had 20 mice each.

Differentiation between contribution of CD4- and CD8-positive cells in augmentation of antimicrobial host defence by liposomal immunomodulators. Next we investigated which subset of T-cells is of major importance in the observed reduction induced by anti-CD3 mAb 17A2, in host defence potentiation after immunomodulation. To this end mice were depleted of CD4- or CD8-positive cells using published dosages and treatment schemes [11]. Depletion of $CD4^+$ cells resulted in a dramatic decrease of host defence potentiation by immunomodulators compared with immunocompetent

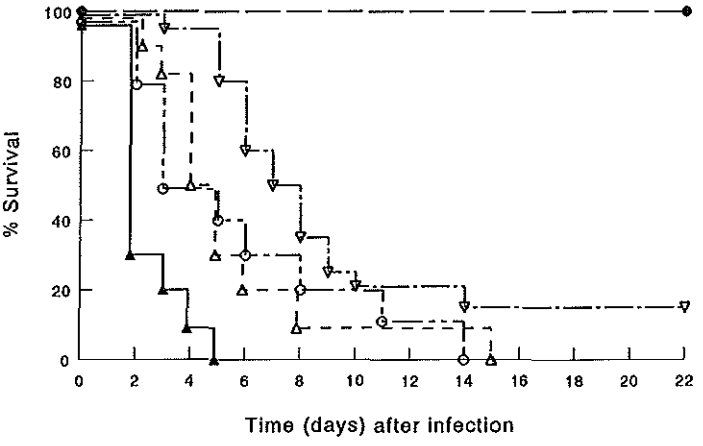

Figure 2. Survival of $CD4^+$ cell depleted mice infected by i.p. inoculation of 10^3 *Klebsiella pneumoniae*. $CD4^+$ cells were depleted by injection of CD4 specific IgG2b mAb. Mice were treated before infection with LE-MTPPE/IFN–γ (▽), LE-MTPPE (△), LE-IFN–γ (○) or PL (▲). Control mice were injected with rat IgG2b anti-ß-galactosidase mAb and treated with LE-MTPPE/IFN–γ (●). All groups had 20 mice each.

controls ($P < .001$) (Fig. 2). In mice treated with LE-MTPPE/IFN–γ survival was reduced from 100% to 15%. Also depletion of $CD8^+$ cells resulted in a dramatic decrease of host defence potentiation by immunomodulators ($P < .001$)(Fig. 3). In mice treated with LE-MTPPE/IFN–γ survival was reduced from 100% in immunocompetent to 5% in CD8-depleted mice. Depletion of $CD3^+$ T-cells as well as depletion of CD4 or CD8 subsets of T-cells had no significant influence on survival of mice treated with PL. In mice treated with LE-MTPPE or LE-IFN–γ depletion of $CD4^+$ or $CD8^+$ cells resulted also in a dramatic reduction of survival ($P < .001$ for both, Fig. 2 and 3 respectively). Taken together, these results indicate that both $CD4^+$ and $CD8^+$ T-cells are of importance in activation of the host defence by liposomal immunomodulators.

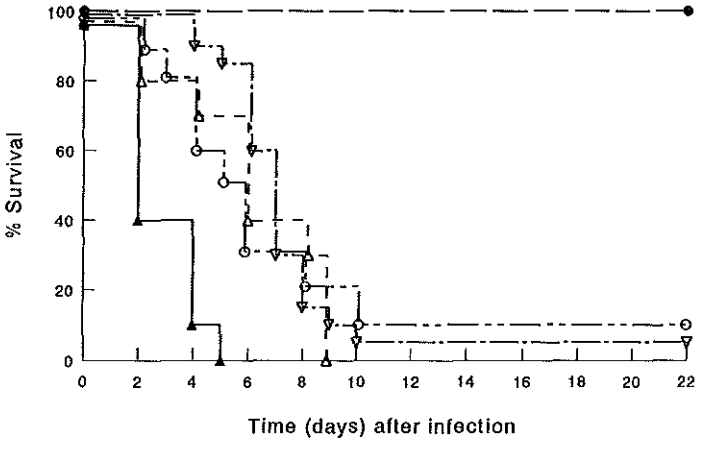

Figure 3. Survival of $CD8^+$ cell depleted mice infected by i.p. inoculation of 10^3 *Klebsiella pneumoniae*. $CD8^+$ cells were depleted by injection of CD8 specific IgG2b mAb. Mice were treated before infection with LE-MTPPE/IFN–γ (▽), LE-MTPPE (△), LE-IFN–γ (○) or PL (▲). Control mice were injected with rat IgG2a anti-ß-galactosidase mAb and treated with LE-MTPPE/IFN–γ (●). All groups had 20 mice each.

Effect of depletion of endogenous IFN–γ on augmentation of antimicrobial host defence by liposomal immunomodulators. Depletion of endogenous IFN–γ by treatment with mAb F3 resulted in a diminished host defence augmentation by the immunomodulators compared with immunocompetent mice ($P < .001$)(Fig. 4). In LE-MTPPE/IFN–γ treated mice depletion of endogenous IFN–γ resulted in a decline of survival from 100% to 15%. Mice treated with LE-MTPPE or LE-IFN–γ showed a more rapid decline in survival by IFN–γ depletion resulting in survival of 15 and 10% respectively.

Effect of administration of IL-10 on augmentation of antimicrobial host defence by liposomal immunomodulators. Also IL-10, produced by intraperitoneal implantated alginate encapsulated IL-10-transfetced cells in mice treated with LE-MTPPE/IFN–γ, induced a decreased host defence: a survival of 20% in IL-10 treated mice compared with 100% in immunocompetent LE-MTPPE/IFN–γ treated mice ($P < .001$) (Fig. 5). Administration of IL-10 producing cells in mice treated with LE-IFN–γ resulted in a comparable decreased survival whereas in LE-MTPPE treated mice a more rapid effect was noticed.

Effect of depletion of endogenous TNF–α on augmentation of antimicrobial host defence by liposomal immunomodulators. Administration of TNF–α-specific rabbit IgG

to LE-MTPPE/IFN–γ treated mice resulted in reduced survival of mice after infection with

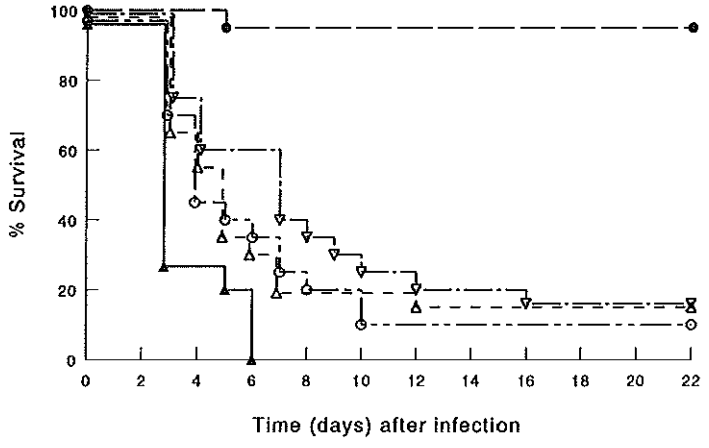

Figure 4. Survival of mice depleted of endogenous IFN–γ, infected by i.p. inoculation of 10^3 *Klebsiella pneumoniae*. IFN–γ was depleted by injection of IFN–γ specific IgG2a mAb. Mice were treated before infection with LE-MTPPE/IFN–γ (▽), LE-MTPPE (△), LE-IFN–γ (○) or PL (▲). Control mice were injected with rat IgG2a anti-ß-galactosidase mAb and treated with LE-MTPPE/IFN–γ (●). All groups had 20 mice each.

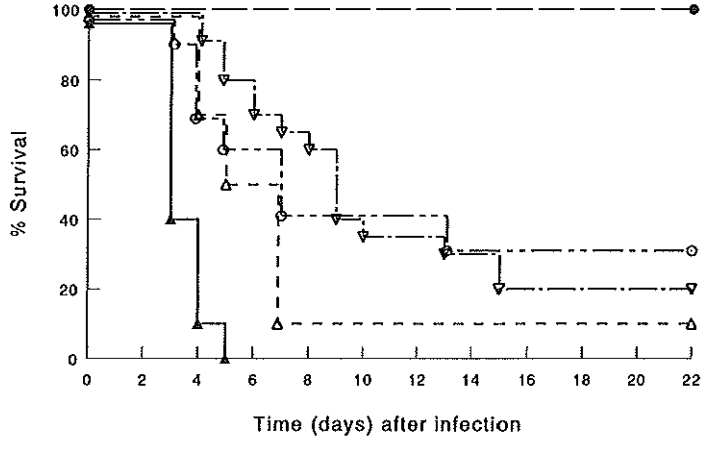

Figure 5. Survival of mice injected ip with IL-10 producing cells in alginate, infected by i.p. inoculation of 10^3 *Klebsiella pneumoniae*. Mice were treated before infection with LE-MTPPE/IFN–γ (▽), LE-MTPPE (△), LE-IFN–γ (○) or PL (▲). Control mice were injected with mock-transfected alginate encapsulated cells and treated with LE-MTPPE/IFN–γ (●). All groups had 20 mice each.

K. pneumoniae ($P < .001$), indicating that stimulation of TNF–α production by macrophages and/or T-cells is also of importance in the described immunomodulation (Fig. 6).

Effect of immunomodulation on MHC-II (Ia) antigen expression of splenic macrophages. The spleen is an important organ in the communication between macrophages and T-cells and a large proportion of the liposomal immunomodulator localizes in the spleen. Moreover, macrophages are considered to be the targets for

immunomodulation, and the liposomes used target mainly to these cells. We therefore examined macrophage activation after immunomodulation in the spleen. Treatment of mice with LE-MTPPE/IFN–γ resulted in enhanced expression of Ia antigen at 4 days after the end of treatment (Fig. 7). Also the complement receptor 3 antigen expression was enhanced upon treatment with immunomodulator already at 12 h after end of treatment (data not shown).

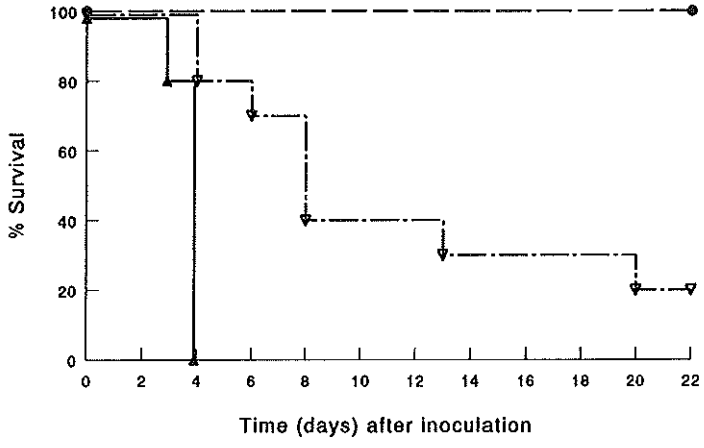

Figure 6. Survival of mice depleted of endogenous TNF-a, infected by i.p. inoculation of 10^3 *Klebsiella pneumoniae*. TNF-a was depleted by injection of TNF-a specific polyclonal rabbit IgG. Mice were treated before infection with LE-MTPPE/IFN–γ (▽) or PL (▲). Control mice were injected with equal amount of rabbit IgG and treated with LE-MTPPE/IFN–γ (●). All groups had 20 mice each.

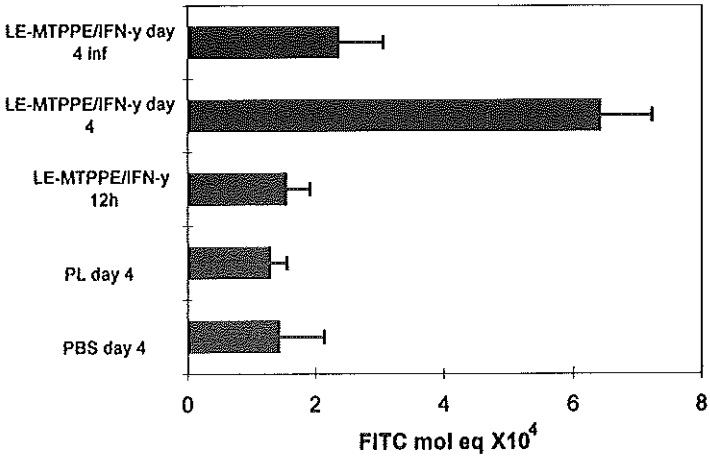

Figure 7. Ia antigen expression on spleen cells measured by flow cytometry in mice treated with LE-MTPPE/IFN–γ, PL or PBS. One group of mice were also infected by i.p. inoculation of 10^3 *Klebsiella pneumoniae* (LE-MTPPE/IFN–γ day 4 inf) 12 h after end of treatment. Spleen cells were examined 12 h and 4 days (day 4) after end of treatment. Each bar represents the average + SD of three mice.

Effect of immunomodulation on Th1 and Th2 cell activity in the spleen. The T-cell depletion studies indicated an important role for T-cells, in particular cell populations producing IFN–γ. This led us to examine the effect of immunomodulation on Th1 and Th2 cell development in the spleen. The activity of T-cell subsets was assessed ex vivo as the amount of IFN–γ (Th1) and IL-4 (Th2) produced after immunomodulation. Treatment of

mice with LE-MTPPE/IFN–γ resulted in T-cells predominantly producing IFN–γ, independently of the culture conditions (Fig. 8). Spleen cells from mice treated with PL produced low levels of IFN–γ. However, spleen cells from LE-MTPPE/IFN–γ treated mice produced high IFN–γ levels when cultured in plain medium or in medium with IL-2 alone, but also when stimulated in the Th1 or Th2 direction ($P < .001$), indicating a strong effect of the immunomodulation on the Th1 population. Whereas T-cells from the spleen produced IFN–γ in high amounts under all culture conditions, production of IL-4 was in most cases below detection level, or even seemed suppressed after immunomodulation. T-cell proliferation was induced by addition of IFN–γ (Th1) or IL-4 (Th2) which explains the high titers especially in Th2 group of graph 2B in figure 8. Taken together, these results indicate a predominant activation by the liposomal immunomodulators of NK cells, CD8 and Th1 cells in the spleen, cells known to produce IFN–γ, and an absence of a significant activation of Th2 cells, which produce IL-4.

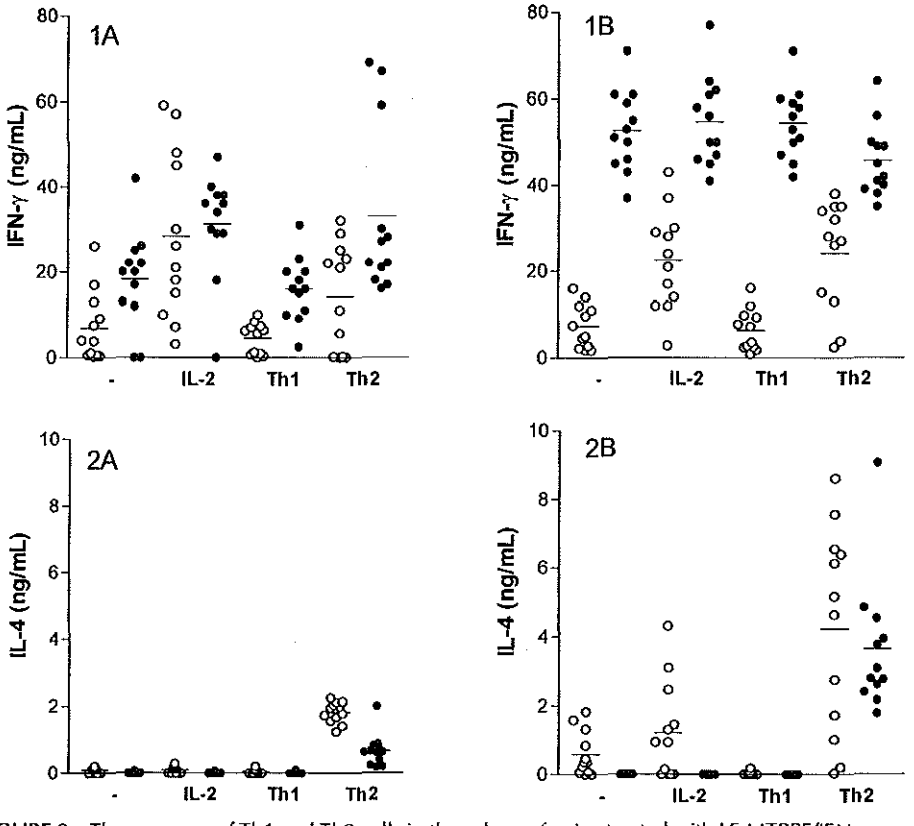

FIGURE 8. The presence of Th1 and Th2 cells in the spleen of mice treated with LE-MTPPE/IFN–γ measured by the production of IFN–γ and IL-4. Three mice were used per treatment group, and cytokine production was determined in 4-fold per mouse. T-cells were isolated 12 h (A) and 4 days (B) after end of treatment. Cells were cultured in anti-CD3 coated plates in the presence of IL-2 (IL-2) or IL-2, IFN–γ and anti-IL-4 (Th1) or IL-2, IL-4 and anti-IFN–γ (Th2) or medium alone (-). Horizontal lines represent averages of the determinations. ○ PL treated controls, and ● LE-MTPPE/IFN–γ treated mice.

DISCUSSION

We have examined the role of T cells in immunomodulation by liposomal MTPPE and IFN–γ in *K. pneumoniae* septicemia in mice. We demonstrate for the first time a key role for T-cells in immunomodulation to Gram-negative septicemia. CD4 and CD8 positive T cells were shown to be important. Furthermore, IFN–γ was shown to be crucial in enhancement of host defence by immunomodulators. Therefore, we hypothesize that especially Th1 cells, CD8 positive cells and NK cells, all known to produce IFN–γ upon stimulation, are activated in this process by liposomal immunomodulator-stimulated macrophages. From experiments with exogenous IL-10, demonstrating that IL-10 can indeed counteract the stimulation of the host defence by the immunomodulators, it is concluded that Th2 cells do not play a significant role in the immunomodulation. Th2 cells may not be stimulated, or are adequately suppressed by IFN–γ. This is supported by our observation that IFN–γ production is increased in the spleen after immunomodulation, indicating a shift to Th1 cells in the spleen upon treatment with LE-MTPPE/IFN–γ.

In mice two types of T helper cells can be distinguished: Type 1 Thelper cells (Th1) producing IL-2, IFN–γ, GM-CSF, but little or no IL-4 and IL-5, and type 2 Thelper cells (Th2) producing IL-3, IL-4, IL-5 and IL-10. One of the aspects of Th1 response is macrophage activation thereby enhancing its capacity to kill intracellular microorganisms. The Th2 response on the other hand leads to macrophage deactivation [reviewed in 19, 20]. The present data indicate an important role for IFN–γ in activation of non-specific host defence by liposomal immunomodulators. In immunocompetent mice NK cells and T cells (Th1 and $CD8^+$) produce IFN–γ upon stimulation [21]. The presumed cell type responsible for the IFN–γ expression in T-cell deficient mice is therefore the NK cell, the only non T-cell reported to produce IFN–γ in vivo [22]. However, depletion of T-cells, both CD4 or CD8, was demonstrated to be detrimental for immunomodulation in mice in our septicemia model, which was shown to depend strongly on IFN–γ. In our model NK cells appeared not able to substantially induce host defence activation in T-cell depleted mice. Taken together, our data show that T-cells are essential for the production of IFN–γ to enhance the host defence upon immunomodulation.

A potent inhibitor of monocyte-macrophage activation is IL-10, produced by Th2 cells and macrophages. IL-10 inhibits production of TNF–α, IL-1 and also IFN–γ by lymphocytes. Moreover, IL-10 is a potent inhibitor of IL-12 production by activated mononuclear cells [reviewed in 23, 24]. IL-12, produced by monocytes/macrophages and B-cells, is required for IFN–γ production in synergy with IL-2 [25, 26], and activation of macrophages by liposomal immunomodulators is expected to induce release of IL-12 resulting in IFN–γ production by activated Th1 and $CD8^+$ cells. Experiments on the role of IL-12 in our model are in progress. Others claim that inhibition of IFN–γ production is primarily due to blocking of the production from accessory cells of IL-12, as well as the co-stimulating cytokine IL-1 [27]. These results support earlier studies that IL-10 does not suppress IFN–γ production by activated lymphocytes directly [28]. The present study shows that exogenous IL-10 has a profound inhibitory effect on survival of mice treated with liposomal immunomodulators, indicating that activation of macrophages most likely results in activation of Th1 cells and NK cells, probably through IL-12, resulting in IFN–γ production [27, 29].

Macrophages are activated by immunomodulators, which is supported by increased Ia antigen expression on these cells upon immunomodulation. The immunomodulators are known to induce TNF–α, presumably produced by activated macrophages, which was shown to be essential for the host defence augmentation by LE-MTPPE/IFN–γ.

Although dramatic reduction of host defence potentiation by immunomodulators was noticed after T-cell depletion, indicating a key role function of these cells in this process, significant enhancement of host defence was still seen in most of the T-cell depleted groups upon treatment with immunomodulators. It appears that next to LE-MTPPE/IFN–γ other (yet unknown) T-cell factors has to be given to obtain sufficient host defence activation in T-cell-deficient hosts.

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REFERENCES

1 ten Hagen TLM, van Vianen W, Bakker-Woudenberg IAJM. Modulation of the non-specific antimicrobial resistance of mice by separate or combined liposome-encapsulated muramyl tripeptide phosphatidylethanolamine and interferon–γ towards *Klebsiella pneumoniae* septicemia. J Infect Dis 1995;171:385-92.

2 Melissen PMB, van Vianen W, Leenen PJM, Bakker-Woudenberg IAJM. Tissue distribution and cellular distribution of liposomes encapsulating muramyltripeptide phosphatidyl ethanolamide. Biotherapy 1994;7:71-8.

3 Fidler IJ, Fogler WE, Kleinerman ES, Saiki I. Abrogation of species specificity for activation of tumoricidal properties in macrophages by recombinant mouse or human interferon-γ encapsulated in liposomes. J Immunol 1985;135:4289-96.

4 Smith MR, Muegge K, Keller JR, Kung H-F, Young HA, Durum SK. Direct evidence for an intracellular role for IFN–γ: microinjection of human IFN–γ induces Ia expression on murine macrophages. J Immunol 1990;144:1777-82.

5 Mehta K, Juliano RL, Lopez-Berestein G. Stimulation of macrophage protease secretion via liposomal delivery of muramyl dipeptide derivatives to intracellular sites. Immunology 1984;51:517-27.

6 Melissen PMB, van Vianen W, Bakker-Woudenberg IAJM. Roles of peripheral leukocytes and tissue macrophages in antimicrobial resistance induced by free or liposome-encapsulated muramyl tripeptide phosphatidylethanolamide. Infect. Immun. 1992;60:2891-7.

7 ten Hagen TLM, van Vianen W, Straathof EAT, Bakker-Woudenberg IAJM. Effect of liposome-encapsulated muramyl tripeptide phosphatidylethanolamine and interferon gamma on *Klebsiella pneumoniae* infection in T-cell-deficient mice and in vitro. In: Masihi KN. Immunotherapy in infections. New York: Marcel Dekker Inc., 1994:225-34.

8 Dijkmans R, Heremans H, Billiau A. Heterogeneity of chinese hamster ovaria cell-produced recombinant murine interferon–γ. J Biol Chem 1987;262:2528-35.

9 Miescher GC, Schreyer M, MacDonald HR. Production and characterization of a rat monoclonal antibody against the murine CD3 molecular complex. Immunol Lett 1989;23:113-8.

10 Cobbold SP, Jayasuriya A, Nash A, Prospero TD, Waldman H. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. Nature 1984;312:548-51.

11 Tomonari K. A rat antibody against a structure functionally related to the mouse T-cell receptor/T3 complex. Immunogenetics 1988;28:455-8.

12 Heremans H, Dijkmans R, Sobis H, Vandekerckhove F, Billiau A. Regulation by interferons of the local inflammatory response to bacterial lipopolysaccharide. J Immunol 1987;138:4175-9.

13 Savelkoul HFJ, van Ommen R, Vossen ACTM, Breedlend EG, Coffman RL, van Oudenaren A. Modulation of systemic cytokine levels by implantation of alginate encapsulated cells. J Immunol Methods 1994;170:185-96.

14 ten Hagen TLM, van Vianen W, Bakker-Woudenberg IAJM. Isolation and characterization of murine Kupffer cells and splenic macrophages. J Immunol Methods 1996;193:81-91.

15 Leenen PJM, de Bruijn MFTR, Voerman JSA, Campbell PA, van Ewijk W. Markers of mouse

macrophage development detected by monoclonal antibodies. J Immunol Methods 1994;174:5-19.
16 Openshaw P, Murphy EE, Hosken NA, Maino V, Davis K, Murphy K, O'Gara A. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. J Exp Med 1995;182:1357-67.
17 Chatelain R, Verkila K, Coffman RL. IL-4 induces a Th2 response in Leishmania major-infected mice. J Immunol 1992;148:1182-1187.
18 Cherwinski HM, Schumacher JH, Brown KD, Mosmann TR. Two types mouse helper T cell clones. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays and monoclonal antibodies. J Exp Med 1987;166:1229-1244.
19 Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;136:2348-57.
20 Romagnani S. Induction of Th1 and Th2 repsonses: A key role for the 'natural' immune response. Immunol Today 1992;13:379-81.
21 Young HA, Hardy KJ. Interferon-γ: Producer cells, activation stimuli, and molecular genetic regulation. Pharmacol Ther 1989;45:137-51.
22 Arase H, Arase N, Saito T. Interferon gamma production by natural killer (NK) cells and NK1.1+ T cells upon NKR-P1 cross-linking. J Exp Med 1996;183:2391-6
23 Moore KW, O'Gara A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. Annu. Rev. Immunol. 1993;11:165-90.
24 Mosmann TR. Properties and Functions of Interleukin-10. Adv Immunology 1994;56:1-26.
25 D'Andrea A, Rengaraju M, Valiante NM, et al. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. J Exp Med 1992;176:1387-98.
26 Chan SH, Kobayashi M, Santoli D, Perussia B, Tranchieri G. Mechanism of IFN-γ induction by natural killer cell stimulatory factor (NKSF/IL-12). J Immunol 1992;148:92-8.
27 D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 (IL10) inhibits human lymphocyte interferon γ-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 1993;178:1041-8.
28 Fiorentino DF, Zlotnik A, Vieira P, et al. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol 1991;146:3444-.
29 Hsieh C-S, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of Th1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 1993;260:547-9.

CHAPTER FOUR

Immunostimulation *in vivo* with liposomal MTPPE plus interferon-γ:
the effect of liposomal co-encapsulated immunomodulators on cell populations in liver, spleen, blood and bone-marrow of mice.

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ABSTRACT

In this study we show the effect of LE-MTPPE/IFN-γ on the hemopoietic composition of liver, spleen, blood and bone marrow in mice. Administration of LE-MTPPE/IFN-γ resulted in the first place in strongly augmented hemopoietic cell numbers in liver and spleen. Especially myeloid cell numbers (i.e. macrophages and granulocytes) were increased in these organs, whereas strongly increased erythropoiesis was also observed in the spleen after treatment. Secondly, treatment with liposomal immunodulators caused increased production of myeloid cells in the bone marrow and enhanced migration of these cells from the bone marrow to the periphery. However, a decline in erythropoiesis was observed in the bone marrow due to treatment, which was compensated by increased erythropoiesis in the spleen.

Based on these results we hypothesize that especially above mentioned hemopoietic effects were induced by liposomal immunomodulators, resulting in increased macrophage and granulocyte cell numbers and an increased erythropoiesis, contributed significantly to increased host defence to infections.

INTRODUCTION

Host resistance towards infections can be enhanced by immunomodulators. Administration of muramyl dipeptides (MDP) or derivatives of MDP, as well as interferon-gamma (IFN–γ) has proven beneficial in activation of host defence to bacterial and viral infections as well as to malignancies (1-4). We have shown that prophylactic administration of liposomal muramyl tripeptide phosphatidylethanolamine (LE-MTPPE), liposomal interferon–γ (LE-IFN–γ) or a combination of these agents co-encapsulated in liposomes (LE-MTPPE/IFN–γ), resulted in survival of mice from a normally lethal bacterial septicemia (2, 5). This effect was predominantly due to an increased clearance of bacteria from the blood (6). These results suggest an important role for stimulated tissue macrophages in liver and spleen in the resistance towards a disseminated infection, as these cells are the primary targets of liposomal immunomodulators (7).

Studies with macrophages exposed to LE-MTPPE, LE-IFN–γ or LE-MTPPE/IFN–γ in vitro demonstrated an enhanced production of nitrogen or oxygen intermediates when stimulated with heat-killed Gram-negative bacteria, suggesting that these agents have their effect by direct activation of the macrophage (2). The enhanced host resistance in vivo to *Klebsiella pneumoniae* was therefore thought to be the result of an increased function of resident tissue macrophages. However, an increased antibacterial activity to *K. pneumoniae* could not be found in vitro when isolated macrophages were stimulated with above mentioned immunomodulators (2). It is speculated that the elevated antimicrobial resistance found in vivo may be the result of an increased number of macrophages in organs, or an effect of the immunomodulators on other cells, rather than direct activation of tissue macrophages. Therefore, increased resistance of mice against bacterial infections after treatment with liposomal immunomodulators might be a result of 1) increased macrophage number due to local proliferation of macrophages as well as recruitment of precursor macrophages from the blood, 2) activation of macrophages and 3) recruitment

or activation of other effector cells (i.e. granulocytes). It was previously shown in our laboratory that depletion of granulocytes before LE-MTPPE treatment resulted in a decline of host defence activation, whereas depletion of macrophages completely abrogated host defence activation by immunomodulators indicating an important role for both these cell types (7, 8).

The aim of this study was to gain more insight into activation of host defence by these liposomal immunomodulators. Therefore, we examined the effect of LE-MTPPE/IFN-γ on the cellular content and hemopoietic composition of liver, spleen, blood and bone-marrow of uninfected mice and mice infected with *K. pneumoniae*. We found increased hemopoiesis in bone marrow towards generation of myeloid cells, which is reflected in blood, spleen and liver. In addition, in spleen also a strongly increased erythropoiesis was demonstrated, compensating reduced erythropoiesis in bone marrow.

MATERIALS AND METHODS

Animals. Specified pathogen free, 11 to 13 week old female C57Bl/Ka mice were used (ITRI-TNO, Rijswijk, The Netherlands).

Bacteria. Klebsiella pneumoniae, capsular serotype 2 (ATCC 43816), was used.

Reagents. Liposome-encapsulated muramyl tripeptide phosphatidylethanolamine (LE-MTPPE) was kindly provided by Ciba-Geigy (Basel, Switzerland). Placebo liposomes (PL) were prepared from a dry lyophilisate composed of phosphatidylcholine and phosphatidylserine in a molar ratio of 7:3 as previously described (2). Recombinant murine IFN-γ was derived from the supernatant fluid of Mick cells, a CHO cell line that carries and expresses an amplified murine IFN-γ cDNA (9). The IFN-γ was purified by affinity chromatography on a anti-rat IFN-γ mAb (DB-1) to a specific activity of 6,000 U/μg. Murine IFN-γ was used at a concentration of $2.5X10^5$ U/ml. Liposomes containing MTPPE and IFN-γ were prepared as described previously by shaking dry lyophilisate with MTPPE in buffer containing IFN-γ (2).

Treatment of mice. Mice were injected with LE-MTPPE/IFN-γ or PL as previously described (2). Briefly, mice were injected i.v. five times every other day with 25 μg MTPPE and 7500 U IFN-γ co-encapsulated in 6.25 mg total lipid. Twelve h after the last dose mice were infected i.p. with *K. pneumoniae* or mock infected with PBS. From the uninfected mice the various organs were examined at day 0 and 4 after mock infection (respectively 12 h and 4 days after end of treatment) and from the infected mice organs were examined at day 4 after infection.

Preparation of cell suspensions. Cell suspensions were prepared from liver and spleen as previously described (10). Briefly, after perfusion of the liver with collagenase A (Boehringer Mannheim, Mannheim, FRG) solution, liver and spleen were excised from mice and the organ weight determined. The organs were incubated in collagenase solution at 35°C for 10 min, and then gently minced and filtered through a nylon gauze (88 μm for spleen and 212 μm for liver). The liver cell suspensions were fractionated on a Nycodenz (Nycomed Pharma AS, Oslo, Norway) gradient to remove the parenchymal cells. The cell suspensions were washed two times, and resuspended at a density of 10^7 cells per ml. The total cell numbers were determined in duplicate in a Bürker's hemocytometer. Bone marrow cells were collected by crunching femora and tibiae in DMEM (GIBCO, Life Technologies Inc. Gaithersburg, MD) supplemented with 5% fetal bovine serum (GIBCO). Clumps of cells were suspended by repeated aspiration through a 23 gauge needle. Subsequently, the bone marrow cell suspension was cleared from bone fragments and debris through a mesh of 30 μm pore size. Blood samples were taken under anaesthesia from the retro-

orbital plexus.

Characterization of isolated cell populations. Cell suspensions were examined by flowcytometry (FACS®, Becton Dickinson, San Jose, CA). Briefly, FACS® analysis was performed with the use of rat anti-mouse monoclonal antibodies (mAb) as hybridoma culture supernatants given in Table 1. Of each cell suspension 25 μl was mixed with 25 μl hybridoma supernatant in 96 well microtiter plates and incubated at room temperature for 10 minutes. Cell suspensions were washed three times with PBS containing 5% FCS and 20 mM sodium azide. Then cells were incubated for 10 minutes at room temperature with FITC-conjugated F(ab)2 fragments of rabbit anti-rat IgG (Cappel, Malvern, PA). Cells were washed three times again and examined. Differential analysis of bone marrow cells was performed using double labeling with ER-MP12 and ER-MP20 (14)

Statistical analysis. Groups were compared using Kruskal-Wallis analysis. *P*-values smaller than 0.05 were considered significant.

Table 1. Specificities of the monoclonal antibodies (mAb) used for flowcytometric analysis of mouse hemopoietic cells.

mAb		Specificity	References
BM8		mature macrophages	b
ER-HR3		subset mature macrophages (spleen)	b
ER-MP12	med[a]:	lymphoid cells (bone marrow)	b
	hi[a]:	blasts (bone marrow)	
ER-MP20	med:	granulocytes	b
	hi:	monocytes	
ER-MP58	med:	erythroid and lymphoid cells	b
	hi:	myeloid cells	
RB6-8C5	med:	granulocyte precursors (Gr-1) (bone marrow)	c
	hi:	granulocytes (Gr-1)	
RA3-6B2		CD45R, B cells, B220	c
KT3		CD3, T cells	c
TER-119		erythroid progenitors	13
30G12		CD45, all leukocytes	c

Monoclonal antibodies were tested on all cell suspensions unless indicated otherwise.
a expression level of cell marker, med is medium and hi is high expression level.
b reviewed in 11
c for references see 12

RESULTS

Effect of LE-MTPPE/IFN-γ on cellular content of liver. Since liver and spleen are the primary organs involved in clearance of bacteria from the blood, we studied the cellular content of these organs after treatment with immunomodulator or placebo. Mice injected with five dosages of LE-MTPPE/IFN-γ demonstrated a pronounced increase in the amount of non-parenchymal cells (NPC) of the liver (Fig. 1A). A 2.5-fold increase was observed 12 h after last dose compared with PL-treated mice ($p < 0.01$). Four days after last dose the

number of NPC was still enhanced in immunomodulator-treated animals compared with PL ($p < 0.05$). Infection of animals with *K. pneumoniae* at 12 h after last dose of immunomodulator did not enhance the amount of NPC further.

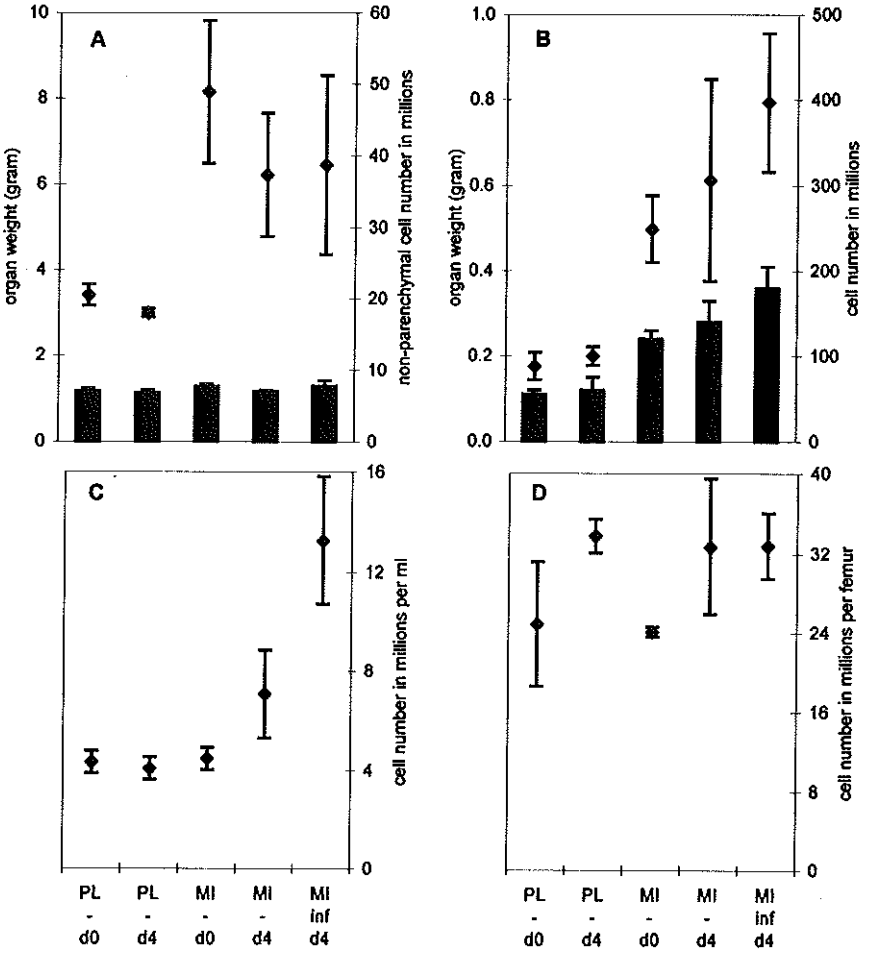

FIGURE 1. Organ weight (bar) and cell number (dot) of liver (A) and spleen (B), and cell number (dot) of blood (C), and bone marrow (D) after treatment with placebo liposome (PL), or liposome co-encapsulated MTPPE and IFN-γ (MI) in uninfected mice (-), or mice infected with *K. pneumoniae* (inf). Organs and tissues were examined 12 h (d0) and 4 days (d4) after the end of treatment. Mean ± SD of triplicate determinations is shown.

FACS analysis of liver NPC demonstrated a general increase of all hemopoietic lineages. Most notably ER-MP58 positive (immature) myeloid cells accounted for the observed increase in NPC at both 12 h and 4 days after end of treatment with LE-MTPPE/IFN–γ (up to 13-fold compared with PL controls, $p < 0.001$)(Fig. 2). A 2-fold increase in granulocytes (RB6-8C5) and Kupffer cells (BM8) was observed in immunomodulator-treated mice compared with PL-treated controls ($p < 0.01$ for both). Immunomodulation also enhanced T and B-cell number significantly, although in infected mice B-cell number was around base value at 4 days after end of treatment. Infection of mice with *K. pneumoniae* after end of treatment did not further increase cell numbers significantly compared with uninfected mice. This indicates that immunomodulation primarily caused an enormous increase in myeloid cell number in liver.

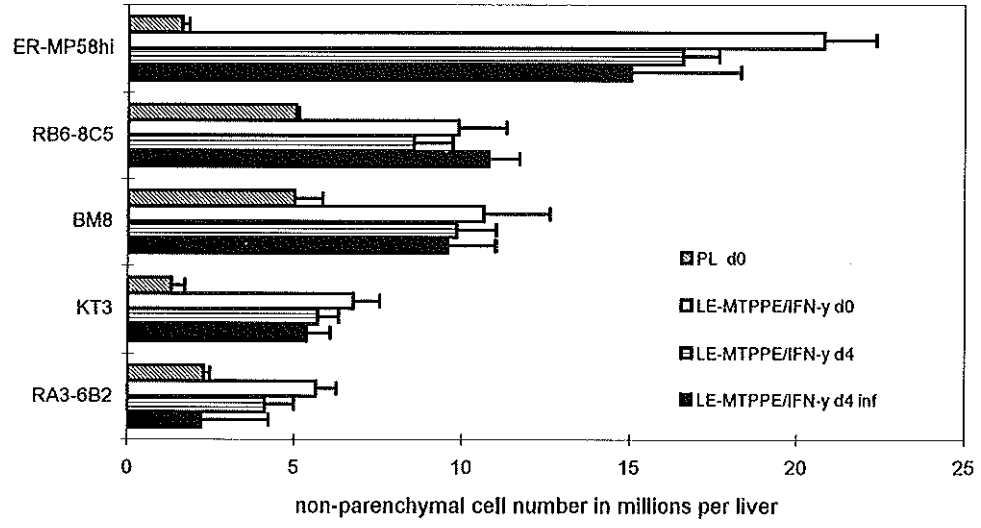

Figure 2. Non-parenchymal cell number in the liver after treatment with placebo liposome (PL), or liposome co-encapsulated MTPPE and IFN–γ (LE-MTPPE/IFN–γ), in uninfected mice (-) or mice infected with *K. pneumoniae* (inf). Livers were examined 12 h (d0) and 4 days (d4) after the end of treatment using the mAb indicated in the graph, which are described in Table 1. Mean ± SD of triplicate determinations is shown.

Effect of LE-MTPPE/IFN–γ on cellular content of the spleen. A dramatic increase of cell number and spleen weight was observed in immunomodulator-treated animals (Fig. 1B). Cell numbers were increased 3-fold at 12 h and 4 days after immunomodulation ($p < 0.005$ and $p < 0.05$, respectively) compared with PL-treated controls. Infection of immunomodulator-treated mice with *K. pneumoniae* resulted in further augmented cell number in spleen up to 4-fold, compared with PL-treated controls ($p < 0.005$).

FACS® analysis of spleen showed that ER-MP58^{hi} positive immature myeloid cells (including colony-forming precursors) were increased 7-fold at 12 h and 15-fold at 4 days after end of treatment with LE-MTPPE/IFN–γ ($p < 0.05$ and $p < 0.001$, respectively) (Fig. 3). A dramatic 40-fold increase of ER-MP58^{hi} myeloid cells compared with PL-treated controls was observed upon infection. This increase concerns also cells of the granulocyte

lineage, as the Gr-1/Ly-6G positive cells (RB6-8C5) showed a 6-fold increase after immunomodulation, and up to 13-fold upon additional infection ($p < 0.001$ for both). Mature macrophages (BM8) were augmented 4.5-fold in spleen at 12 h and 11-fold at 4 days after end of treatment ($p < 0.005$ and $p < 0.005$, respectively), which was confirmed by ER-HR3 (majority of splenic red pulp macrophages). ER-HR3 positive cells were also increased in *K. pneumoniae* infected mice ($p < 0.01$).

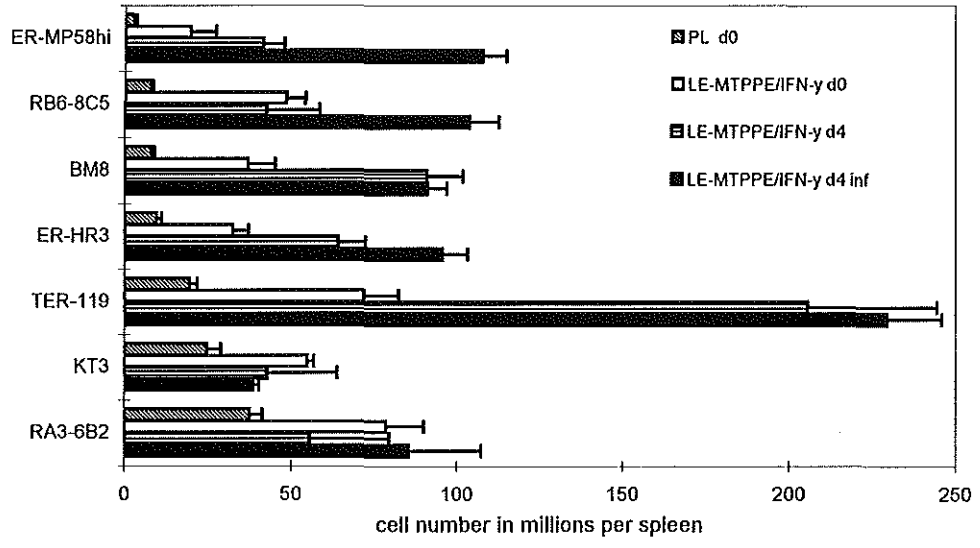

Figure 3. Cell number in the spleen after treatment with placebo liposome (PL), or liposome co-encapsulated MTPPE and IFN-γ (LE-MTPPE/IFN-γ) in uninfected mice (-) or mice infected with *K. pneumoniae* (inf). The spleen was examined 12 h (d0) and 4 days (d4) after the end of treatment using the mAb indicated in the graph, which are described in Table 1. Mean ± SD of triplicate determinations is shown.

The number of erythroblasts (TER-119 positive cells) were strongly enhanced up to 3.6- fold at 12 h ($p < 0.001$), and 10-fold at 4 days after immunomodulation ($p < 0.005$). Thus macrophages and erythroblasts showed a continuing increase in cell number from 12 h to 4 days after last dose with immunomodulator. However, infection of mice did not change TER-119 positive cell numbers compared with uninfected mice. Minor changes were found in T and B-cell numbers. Taken together, these findings show a strong increase in myeloid and erythroid cell number in spleen induced by LE-MTPPE/IFN-γ, indicating augmented local erythropoiesis and localization of myeloid cells, possibly accompanied by local myelopoiesis.

Effect of LE-MTPPE/IFN-γ on cellular content of blood. Since blood plays an important intermediary role in cell recruitment, we studied the cellular content of blood after treatment with immunomodulator or placebo. Cell numbers in blood were slightly enhanced 2-fold at 4 days after end of treatment in immunomodulator-treated mice compared with PL- treated controls ($p = 0.06$) (Fig. 1C). However, when mice were infected with *K. pneumoniae* the number of leukocytes was augmented significantly at 4

days after end of treatment ($p < 0.05$).

FACS® analysis performed on blood cells demonstrated that especially granulocytes (RB6-8C5 9-fold, $p < 0.01$) and monocytes (ER-MP20^{hi} 11-fold, $p < 0.05$) accounted for this increase in leukocyte number in blood upon infection compared with uninfected PL-treated controls (Fig. 4). These findings show that treatment with LE-MTPPE/IFN–γ increased peripheral blood leukocyte number at 4 days after end of treatment, but more pronounced increase of monocytes and granulocytes was seen in infected mice.

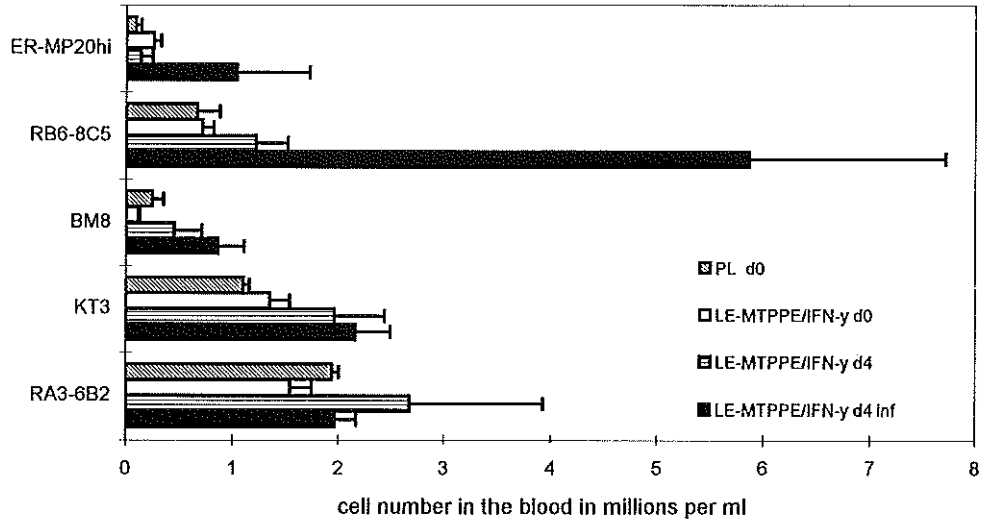

Figure 4. Cell number in the blood per ml after treatment with placebo liposome (PL), or liposome co-encapsulated MTPPE and IFN–γ (LE-MTPPE/IFN–γ) in uninfected mice (-) or mice infected with *K. pneumoniae* (inf). Blood samples were examined 12 h (d0) and 4 days (d4) after the end of treatment using the mAb indicated in the graph, which are described in Table 1. Mean ± SD of triplicate determinations is shown.

Effect of LE-MTPPE/IFN–γ on cellular content of bone marrow. Since bone marrow is very important in hemopoiesis, we studied the cellular content after treatment with immunomodulator or placebo. In contrast with other tissues treatment with LE-MTPPE/IFN–γ decreased total cell amount 1.4-fold to 24.1 ± 0.5 at 12 h after end of treatment, which is comparable with PL (Fig. 1D). However, cell numbers returned to control values at 4 days after end of treatment in all groups to values comparable with buffer treated animals: 32.5 ± 5.9 cells per femur.

FACS® analysis demonstrated 1.6-fold increase in total myeloid cells (ER-MP58^{hi}) at 4 days after end of treatment with LE-MTPPE/IFN–γ compared with PL-treated controls ($p < 0.01$)(Fig. 5). Infection of mice had no further enhancing effect. Treatment with LE-MTPPE/IFN–γ induced 1.3-fold increase of monocytes after initial decline compared with PL treated mice ($p < 0.05$)(Table 2).

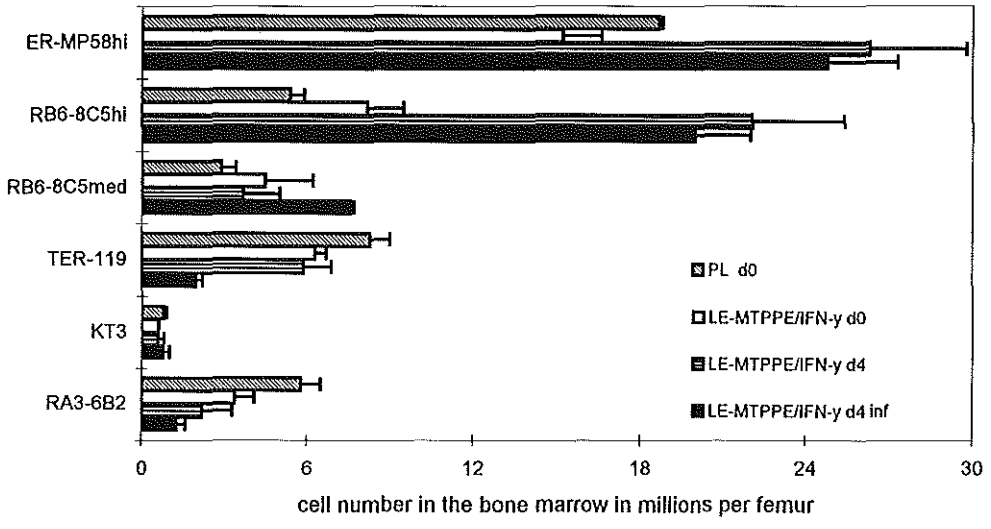

Figure 5. Cell number in bone marrow per femur after treatment with placebo liposome (PL), or liposome co-encapsulated MTPPE and IFN-γ (LE-MTPPE/IFN-γ), in uninfected mice (-) or mice infected with *K. pneumoniae* (inf). Tissues were examined 12 h (d0) and 4 days (d4) after the end of treatment using the mAb indicated in the graph, which are described in Table 1. Mean ± SD of triplicate determinations is shown.

Table 2. Cell populations in the bone marrow of mice in absolute number per femur ($X10^6$) and as percentage of the buffer-treated controls (between parentheses).

	Cell markers and populations[a]				
Treatment[b]	$12^{med}20^-$ lymphocytes	12^-20^- erythroid cells	12^+20^+ mixed blasts	12^-20^{med} granulocytes[c]	12^-20^{hi} monocytes
PBS	5.5 (100)	11.7 (100)	3.3 (100)	10.0 (100)	2.5 (100)
PL d0	5.8 (104.4)	10.9 (93.2)	2.9 (88.8)	6.5 (64.6)	2.3 (92.9)
PL d4	5.2 (94.0)	13.9 (118.8)	3.7 (113.3)	10.3 (102.7)	2.5 (102.0)
LE-MTPPE/IFN-γ d0	2.7 (49.2)	6.4 (54.3)	3.9 (118.4)	9.0 (89.8)	2.0 (80.8)
LE-MTPPE/IFN-γ d4	2.2 (39.3)	7.0 (59.8)	3.6 (111.2)	19.6 (195.3)	3.2 (130.3)
LE-MTPPE/IFN-γ d4 inf	1.7 (30.7)	5.9 (50.4)	6.0 (183.7)	15.5 (154.6)	3.3 (133.3)

a Double labeling with mAbs ER-MP12 and ER-MP20 which are described in Table 1, of which an example is given in figure 6.

b Treatment with placebo liposomes (PL), or liposome co-encapsulated MTPPE and IFN-γ (LE-MTPPE/IFN-γ) in uninfected mice or mice infected with *K. pneumoniae* (inf).

c In accordance with virtual identical data found for Gr-1^{hi} cells.

Granulocyte cell numbers were increased 2-fold as measured by ER-MP12 and ER-MP20 double labeling (ER-$MP12^-20^{med}$ cell population), after initial decline, at 4 days after end of treatment with LE-MTPPE/IFN-γ compared with PBS treated controls ($p < 0.005$)(Table 2). Infection of mice with *K. pneumoniae* after immunomodulation did not increase granulocyte number further. A 1.6-fold and 1.3-fold increase of granulocyte progenitor cells (RB6-$8C5^{med}$) was seen at 12 h and 4 days after treatment with LE-

MTPPE/IFN–γ respectively compared with PL-treated mice (not significant)(Fig. 5). Infection, however, increased granulocyte precursor cells significantly up to 3.0-fold compared with uninfected PL- treated mice ($p < 0.05$). However, ER-MP12$^+$20$^+$ cell population (mixed blast cells) failed to demonstrate a similar increase of these cells after treatment with LE-MTPPE/IFN–γ as compared with PL-treated controls (Table 2), most likely because these granulocyte progenitors bear the mature ER-MP12$^+$20^{med} phenotype. Only in infected animals a 1.8-fold increase of the blast cell population was observed when compared with PL-treated controls (Table 2 and Fig. 6).

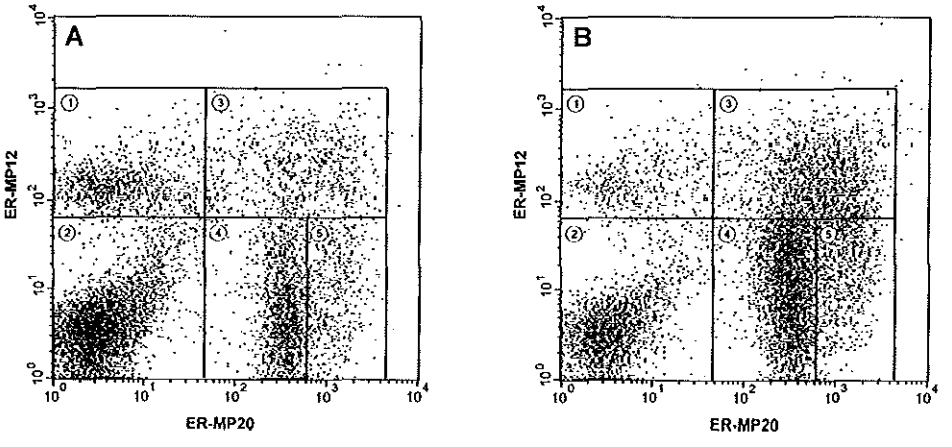

Figure 6. Flowcytometric analysis of bone marrow with mAb ER-MP12 and ER-MP20 double labeling. This graph shows representative data of three pooled mice treated with placebo liposomes (A), or three pooled mice treated with liposome co-encapsulated LE-MTPPE and IFN–γ and infected with *K. pneumoniae* (B). The bone marrow was examined at 4 days after end of treatment. The bone marrow can be divided into five cell populations indicated in the graph: 1) lymphocytes, 2) erythroid cells, 3) mixed blasts, 4) granulocytes, and 5) monocytes.

Reduction of erythroid cells (TER-119, ER-MP12$^+$20$^-$) was observed after treatment with LE-MTPPE/IFN–γ at 12 h and 4 days after end of treatment (reduction to 72% and 67% of control level respectively, $p < 0.05$ for both). Infection of mice even further decreased erythroid cells 4.3-fold compared with PL-treated controls ($p < 0.001$)(Fig. 5) (Table 2). The reduction is due to the liposome-encapsulated immunomodulators as PL-treatment had no significant effect on erythroid cell numbers in bone marrow.

T-cells (KT3) were found in bone marrow of all groups in comparable amounts (Fig. 5). However, the number of B-cells (RA3-6B2) declined 1.5-fold and 2.4-fold respectively at 12 h and 4 days after end of treatment with LE-MTPPE/IFN–γ ($p < 0.05$ and $p < 0.01$, respectively)(Fig. 5). Infection induced further decline in B-cell number in mice: 3.8-fold reduction compared with uninfected PL-treated controls ($p < 0.01$). These findings are in agreement with detection of total lymphoid cells (ER-MP12^{med}20$^-$) (Table 2).

Taken together these data demonstrate that treatment with LE-MTPPE/IFN–γ results in an augmented production of myeloid cells in bone marrow after initial depletion; the erythroid cell production however, is simultaneously suppressed.

DISCUSSION

Enhancement of non-specific host defence by MDP or derivatives and IFN–γ has been shown in numerous studies (4, reviewed in 15). More recently it was shown in our laboratory that multiple treatment with liposomal co-encapsulated MTPPE and IFN–γ substantially increased antimicrobial host resistance, resulting in 100% survival of mice from a normally lethal *K. pneumoniae* septicemia (2). In the present paper we studied this effect of immunomodulation with LE-MTPPE/IFN–γ in more detail. Thereto cell populations in liver, spleen, blood, and bone marrow were quantitated and characterized.

Here we show that treatment with LE-MTPPE/IFN–γ resulted in strongly augmented production of myeloid cells in bone marrow, which is reflected in peripheral organs and blood. Also in liver and spleen an according rise in number of myeloid cells is demonstrated, which probably results from both local proliferation and recruitment from bone marrow. In spleen a strong local erythropoiesis is induced by the treatment, which compensates for the observed decline in bone marrow.

The most important effect of treatment in liver is the increase in myeloid cells: both macrophage and granulocyte cell numbers were increased. The number of immature macrophages was most augmented, suggesting recruitment of immature cells from bone marrow. Likewise, it was found by others that treatment of mice with biological response modifiers increased the number of NK cells in liver, due to redistribution from spleen, or recruitment from bone marrow (16).

In spleen a pronounced increase in myeloid and erythroid cells was found after treatment with LE-MTPPE/IFN–γ, which resulted in significant augmentation of spleen weight and cell number. The present data show that both granulocyte and macrophage cell numbers were augmented, most likely resulting from maturation of the expanding granulocyte-macrophage progenitors after treatment with LE-MTPPE/IFN–γ. Experiments using LE-MTPPE alone in irradiated mice also demonstrated an increase in cell number in spleen (17), but not in mice treated with MDP (18). However, MDP alone induced an expansion of the granulocyte progenitors in spleen (19). The improved activity of MTPPE compared with MDP is most likely due to improved targeting of MTPPE to macrophages by liposomal encapsulation. The increased splenic erythropoiesis described by us most likely resulted from local proliferation of erythroid progenitors, which are increased by mobilization of stem cells to the spleen, as our data shows bone marrow erythropoiesis is strongly reduced. Increase in erythropoiesis was not found in liver. Mobilization of cells from bone marrow to spleen was also demonstrated by Fedoročko et al. in irradiated mice after treatment with LE-MTPPE (20).

In blood less pronounced differences were found between treated mice and control mice. However, when mice were infected after treatment with LE-MTPPE/IFN–γ strongly augmented monocyte and granulocyte numbers were observed. The cell population found in circulation is probably a reflection of the increased hemopoietic activity of bone marrow and spleen.

Strikingly, treatment of mice with LE-MTPPE/IFN–γ resulted initially in a decline in the cellular content of bone marrow. Since this effect was also seen with placebo liposomes, we therefore designated this as a liposome effect. However, values returned to control levels after 4 days in PL treated mice, whereas LE-MTPPE/IFN–γ treated mice

showed a further increase in the cellular content. Probably liposomes by themselves, as particulate stimulants, already trigger bone marrow to release cells to the periphery.

The most important effect of treatment with LE-MTPPE/IFN–γ on bone marrow was the induction of myeloid cell generation. Especially production of granulocytes and granulocyte progenitors, and to lesser extent monocytes, was augmented. These results are in agreement with increased granulocyte cell numbers in irradiated mice after treatment with LE-MTPPE (17). However, treatment with LE-MTPPE/IFN–γ also resulted in depression of erythropoiesis in bone marrow. This was even more pronounced when mice were subsequently infected. Also the number of lymphoid cells (B-cells) were reduced in bone marrow after treatment. From the present data it can be concluded that treatment with LE-MTPPE/IFN–γ resulted in a shift in bone marrow hemopoiesis towards generation of myeloid cells, whereas erythropoiesis declined. The spleen seems to compensate for bone marrow drainage or failure to keep up erythropoiesis.

Studies with MDP demonstrated an in vivo hemopoietic response characterized by a rise in level of monocyte-macrophage colony-stimulating activity (CSA) in serum and an expansion of granulocyte-macrophage progenitors in spleen (19, 21, 22). Also MTPPE and IFN–γ induce production of cytokines and growth factors, which exhibit hemopoiesis stimulating activities. The results presented here indicate that systemic processes are activated through LE-MTPPE/IFN–γ activated macrophages resulting in the observed hemopoietic effects, both in bone marrow as well as in other organs studied.

Previous studies indicated that functional activation of tissue macrophages with immunomodulators does not always result in enhanced antimicrobial activity. In vitro activation of macrophages with LE-MTPPE/IFN–γ failed to increase phagocytosis of *K. pneumoniae* (2). These results seem to be in discrepancy with in vivo observations showing that depletion of macrophages is detrimental for host defence activation by LE-MTPPE in a *K. pneumoniae* septicemia (8). From the present study it becomes clear that treatment with LE-MTPPE/IFN–γ next to macrophage activation also induces strong hemopoiesis in bone marrow and spleen. We think that increase in cell number of macrophages and granulocytes is a more important factor in the observed increase in host defence than just activation of these cells. A probable explanation for the observed effects is that a rise in hemopoietic growth factors in serum, in combination with certain cytokines (TNF-α, IFN–γ, IL-1 and IL-6), results in 1) augmented myelopoiesis in bone marrow and erythropoiesis in spleen, 2) an augmented migration of especially myeloid cells to periphery, and 3) an increased local proliferation of macrophages in spleen and liver. Together, these effects may account for the profound increase in antimicrobial host defence, which is seen in our infection model after treatment with LE-MTPPE/IFN–γ.

REFERENCES

1 Fidler, I.J. (1992) Systemic macrophage activation with liposome-entrapped immunomodulators for therapy of cancer metastasis. *Res. Immunol.* **143**, 199-204.

2 ten Hagen, T.L.M., van Vianen, W., Bakker-Woudenberg, I.A.J.M. (1995) Modulation of the non-specific antimicrobial resistance of mice by separate or combined liposome-encapsulated muramyl tripeptide phosphatidylethanolamine and interferon-γ towards *Klebsiella pneumoniae* septicemia. *J. Infect. Dis.* **171**, 385-392.

3 Koff, W.C., Showalter, S.D., Hampar, B., Fidler, I.J. (1985) Protection of mice against fatal herpes

simplex type 2 infection by liposomes containing muramyl tripeptide. *Science* **228**, 495-497.

4 Murray, H.W. (1994) Interferon-gamma and host antimicrobial defense: Current and future clinical applications. *Am. J. Med.* **97**, 459-467.

5 Melissen, P.M.B., van Vianen W., Bakker-Woudenberg I.A.J.M. (1994) Treatment of Klebsiella septicemia in normal and leukopenic mice by liposome-encapsulated muramyl tripeptide phosphatidylethanolamide. *Antimicrob. Agents Chemother.* **38**, 147-150.

6 Melissen, P.M.B., van Vianen, W., Rijsbergen, Y., Bakker-Wudenberg, I.A.J.M. (1991) Free versus liposome-encapsulated muramyl tripeptide phosphatidylethanolamine in treatment of experimental *Klebsiella pneumoniae* infection. *Infect. Immun.* **60**, 95-101.

7 Melissen, P.M.B., van Vianen, W., Bakker-Woudenberg, I.AJ.M. (1992) Roles of peripheral leukocytes and tissue macrophages in antimicrobial resistance induced by free or liposome-encapsulated muramyl tripeptide phosphatidylethanolamide. *Infect. Immun.* **60**, 2891-2897.

8 Melissen, P.M.B., W. van Vianen, and I.A.J.M. Bakker-Woudenberg. (1994)Treatment of Klebsiella Septicemia in Normal and Leukopenic Mice by Liposome-Encapsulated Muramyl Tripeptide Phosphatidylethanolamide. *Antimicrob. Agents Chemother.* **38**,147-150.

9 Dijkmans, R., Heremans, H., Billiau, A. (1987) Heterogeneity of Chinese hamster ovaria cell-produced recombinant murine interferon–γ. *J. Biol. Chem.* **262**, 2528-2535.

10 ten Hagen, T.L.M., van Vianen, W., Bakker-Woudenberg, I.A.J.M. (1996) Isolation and chracterization of murine Kupffer cells and splenic macrophages. *J. Immunol. Methods* **193**, 81-91.

11 Leenen, P.J.M., de Bruijn, M.F.T.R., Voerman, J.S.A, Campbell, P.A., Van Ewijk, W. 1994. Markers of mouse macrophage development detected by monoclonal antibodies. *J. Immunol. Methods* **174**, 5-19.

12 Slieker, W.A.T., J.C.M. van der Loo,de Rijk-de Bruijn, M.F.T.R., Godfrey, D.I., Leenen, P.J.M., Van Ewijk, W. (1993) ER-MP12 antigen, a new cell surface marker on mouse bone marrow cells with thymus-repopulating ability: II. Thymus-homing ability and phenotypic characterization of ER-MP12-positive bone marrow cells. *Int. Immunol.* **5**, 1099-1107.

13 Ikuta, K., Kina, T., MacNeil, I., Uchida, N., Peault, B., Chien, Y., Weissman, I.L. (1990) A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell* **62**, 863-874.

14 De Bruijn, M.F.T.R., Slieker, W.A.T, van der Loo, J.C.M.,Voerman, J.S.A., Van Ewijk, W., Leenen, P.J.M. (1994) Distinct mouse bone marrow macrophage precursors identified by differential expression of ER-MP12 and ER-MP20 antigens. *Eur. J. Immunol.* **24**, 2279-2284.

15 Audibert, F., Leclerc, C., Chedid, L. (1985) Muramyl peptides as immunopharmacological response modifiers. In *Biological Response Modifiers* (P.F. Torrence, ed) Academic Press, NY, NY, p. 307-327.

16 Wiltrout, R.H., Pilaro, A.M., Gruys, M.E., Taldmadge, J.E., Longo, D.L., Ortaldo, J.R., Reynolds, C.W. (1989) Augmentation of mouse liver-associated natural killer activity by biologic response modifiers occurs largely via rapid recruitment of large granular lymphocytes from the bone marrow. *J. Immunol.* **143**, 372-378.

17 Macková, N.O., Fedoročko, P. (1993) Pre- and postirradiation hemopoietic effects of liposomal muramyl tripeptide phosphatidylethanolamine (MTPPE) administered to C57bl/6 mice before irradiation. *Neoplasma* **40**, 379-385.

18 Wuest, B., Wachsmuth, E.D. (1982) Stimulatory effect of N-acetyl muramyl dipeptide in vivo: proliferation of bone marrow progenitor cells in mice. *Infect. Immun.* **37**, 452-462.

19 Galelli, A., Chedid, L. (1986) Modulation of myelopoiesis in vivo by synthetic adjuvant-active muramyl peptides: Induction of colony-stimulating activity and stimulation of stem cell proliferation. *Infect. Immun.* **42**, 1081-1085.

20 Fedoročko P. (1994) Liposomal muramyl tripeptide phosphatidylethanolamine (MTPPE-PE) promotes heamopoietic recovery in irradiated mouse. *Int. J. Radiat. Biol.* **65**, 465-475.

21 Broudy, V.C., Kaushansky, K., Shoemaker, S.G., Aggarwal, B.B., Adamson, J.W. (1990) Muramyl dipeptide induces production of hemopoietic growth factors in vivo by a mechanism independent of tumor necrosis factor. *J. Immunol.* **144**, 3789-3794.

22 Galelli, A., Chedid, L. (1986) Induction of colony-stimulating activity (CSA) by a synthetic muramyl peptide (MDP): Synergism with LPS and activity in C3H/HeJ mice and in endotoxin-tolerized mice. *J. Immunol.* **137**, 3211-3215.

CHAPTER FIVE

Expression of Immuno-regulatory Cytokines Determined by mRNA-RT-PCR After Immunomodulation Using Liposome-encapsulated MTPPE/IFN-γ in Mice.

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ABSTRACT

Mice were treated with liposome-coencapsulated immunomodulators muramyl tripeptide phosphatidylethanolamine (MTPPE) and IFN–γ (LE-MTPPE/IFN–γ), and expression of mRNA in liver and spleen was examined. We demonstrated that five-fold injection of LE-MTPPE/IFN–γ results in increased mRNA expression of IL-2 and IL-10 in liver, and an increased IL-10 mRNA expression in spleen. Strikingly, neither in liver nor spleen was expression of TNF–α nor IL-6 mRNA not observed. The IL-10 mRNA expression was increased in the liver and spleen at 96 h after end of treatment, whereas at 12 h after end of treatment in liver and spleen a decline in IL-10 mRNA expression was observed in LE-MTPPE/IFN–γ as compared with controls. Administration of LE-MTPPE/IFN–γ was previously shown by us to result in pronounced host defence activation. The present results indicate that T-cells are involved in that process as shown by production of IL-2 mRNA. Th2 cells are activated especially in a late stadium after immunomodulation as shown by IL-10 mRNA expression. The absence of TNF–α mRNA expression might indicate that macrophage activation by immunomodulators is transient (within 12 after injection) or takes place only after the first rounds of injections with immunomodulator.

INTRODUCTION

In several models of infection, activation of the non-specific host defence system by muramyl peptides and IFN–γ has been convincingly shown to be effective. We have previously demonstrated that prophylactic treatment of mice to a lethal *Klebsiella pneumoniae* septicemia with liposome-encapsulated muramyl tripeptide phosphatidylethanolamine (LE-MTPPE) or interferon–γ (LE-IFN–γ), or both immunomodulators co-encapsulated in liposomes (LE-MTPPE/IFN–γ) could augment survival substantially (1). Macrophages were shown to exhibit intracellular receptors for both immunomodulators, and are considered the primary target for liposomal immunomodulators (2-5). It was demonstrated that MPS cells are of major importance in the observed host defence potentiation with LE-MTPPE in vivo (6).

However, in vitro increased phagocytosis of *K. pneumoniae* was not observed after exposure of macrophages to immunomodulators (1). These data suggest that activation of single MPS cells is not sufficient to obtain effective killing of this microorganism. Alternatively, macrophages are lacking important co-stimulating signals under in vitro conditions. Therefore, it is speculated that activation of macrophage, or MPS as a whole, is also influenced by other cells (i.e. T-cells). Evidence for this comes from studies in which mice were depleted of T-cells before treatment with liposome-encapsulated immunomodulators. T-cell depletion resulted in a dramatic reduction of immunomodulator-induced antimicrobial host defence potentiation towards *K. pneumoniae* infection (ten Hagen et al; manuscript submitted for publication). These observations implicated an important role for T-cells in potentiation of host defence by liposomal immunomodulators. Moreover, in the same study it was shown that especially

IFN–γ production by T-cells is important, whereas IL-4 or IL-10 did not seem to play a role of significance.

In vivo studies on the mechanism of host defence activation by immunomodulators revealed that administration of five dosages of LE-MTPPE/IFN–γ resulted in profound increase in the number of macrophages and granulocytes in liver and spleen (ten Hagen et al; manuscript submitted for publication). A strongly increased erythropoiesis was also observed in spleen after treatment, and an increased production of myeloid cells in bone marrow. These observation indicate that immunomodulation not only results in direct activation of tissue macrophages, but also in augmentation of hemopoiesis. We speculate that this is the result of a cytokine network, activated by liposomal immunomodulators, which involves macrophages, but also T-cells and other important regulatory cells.

In the present paper induction of mRNA expression of important regulatory cytokines by LE-MTPPE, LE-IFN–γ or LE-MTPPE/IFN–γ was studied in vivo. To this end, mice were treated with immunomodulators after which liver and spleen were removed and mRNA expression quantified after PCR.

MATERIALS AND METHODS

Animals. Specified pathogen free, 11 to 13 week old female C57Bl/Ka mice were used (ITRI-TNO, Rijswijk, The Netherlands).

Bacteria. Klebsiella pneumoniae, capsular serotype 2 (ATCC 43816), was used.

Reagents. Liposome-encapsulated muramyl tripeptide phosphatidylethanolamine (LE-MTPPE) was kindly provided by Ciba-Geigy (Basel, Switzerland). Placebo liposomes (PL) were prepared from a dry lyophilisate composed of phosphatidylcholine and phosphatidylserine in a molar ratio of 7:3 as previously described (1). Recombinant murine IFN–γ was derived from supernatant fluid of Mick cells, a CHO cell line that carries and expresses an amplified murine IFN–γ cDNA (7). IFN–γ was purified by affinity chromatography on an anti-rat IFN–γ mAb (DB-1) to a specific activity of 6,000 U/μg. Murine IFN–γ was used at a concentration of $2.5X10^5$ U/ml. Liposomes containing MTPPE and IFN–γ were prepared as described previously by shaking dry lyophilisate with MTPPE in buffer containing IFN–γ (1).

Treatment of mice. Mice were injected with LE-MTPPE/IFN–γ or PL as previously described (1). Briefly, mice were injected i.v. five times every other day with 25 μg MTPPE and 7500 U IFN–γ co-encapsulated in 6.25 mg total lipid. From mice liver and spleen were examined at 12 h and 96 h after end of treatment.

Preparation of cell suspensions. Cell suspensions were prepared from liver and spleen as previously described (8). Briefly, after perfusion of the liver with collagenase A (Boehringer Mannheim, Mannheim, FRG) solution, liver and spleen were excised from mice and the organ weight determined. Organs were incubated in collagenase solution at 35°C for 10 min, and then gently minced and filtered through a nylon gauze (88 μm for spleen and 212 μm for liver). Liver cell suspensions were fractionated on a Nycodenz (Nycomed Pharma AS, Oslo, Norway) gradient to remove parenchymal cells. Cell suspensions were washed two times, and resuspended at a density of 10^7 cells per ml. Total cell numbers were determined in duplicate in a Bürker's haemocytometer.

Semi-quantitative PCR on mRNA from liver and spleen.

RNA isolation. RNA was isolated from non-parenchymal liver cell or spleen cells using a single-step method according to Chomczinski et al. (9). RNA was stored at -80°C until further use.

RT-PCR. Reverse transcription (RT) of total RNA was performed with avian myeloblastosis virus reverse transcriptase in RT buffer with RNase inhibitor, an oligo(dT)$_{15}$ primer, a deoxynucleoside triphosphate mixture and spermidine in a final volume of 20 μl. Prepared cDNA was subjected to PCR with primers for HPRT, TNF–α, IFN–γ, IL-2, IL-4, IL-6, and IL-10. Briefly, 10 μl volumes of the cDNAs were amplified in the presence of 5' and 3' primers, deoxynucleoside triphosphate mixtures and *Taq* DNA polymerase in a final volume of 40 μl. The reaction mixture was overlaid with mineral oil, and PCR was performed in a Perkin-Elmer Cetus Thermal Cycler. The first cycle consisted of 1.5 min of denaturation at 94°C and was followed by 30, 35, 40 or 45 cycles of 30 s 94°C, 30 s 55°C and 60 s 72°C. The reaction product was visualized by electrophoresis of 20 μl reaction mixture in a 2% agarose gel containing ethidium bromide. Gels were examined with UV light and photographed. FX174 DNA molecular size markers (Biolabs, Beverly, MA) were run in parallel. RT-PCR was performed on 3 to 6 different liver or spleen cell isolates per experimental group. The PCR products were quantified by comparison with positive controls (expression level 5.0) and negative controls (expression level 1.0), resulting in 5 expression levels (5.0, 4.0, 3.0, 2.0 and 1.0).

Statistical analysis. Statistical analysis was performed using the non-parametric Kruskal-Wallis test.

RESULTS

Isolation of non-parenchymal liver cells and spleen cells. Around 114X10^6 cells were isolated from liver of PBS treated mice (Table 1). The number of non-parenchymal cells increased 1.4-fold in mice at 96 h after treatment with LE-MTPPE/IFN–γ. From spleen of PBS controls 146X10^6 cells could be isolated. Cell number increased 2- and 4-fold respectively 12 h and 96 h after treatment with LE-MTPPE/IFN–γ. Both in liver and spleen administration of PL had no significant effect on cell numbers. These findings are in close agreement with earlier observations in our laboratory (ten Hagen et al; manuscript submitted for publication).

Table 1. Non-parenchymal liver cell numbers and spleen cell numbers of mice after treatment with LE-MTPPE/IFN–γ in absolute number (X10^6) per organ.

	Liver		Spleen	
	Time after end of treatment			
Treatment	12 h	96 h	12 h	96 h
LE-MTPPE/IFN–γ	127	171	292	618
PL	121	128	140	215
PBS	114	120	146	154

Mice were given five dosages of LE-MTPPE/IFN–γ, PL or PBS, after which livers and spleens were excised and the cell numbers determined. Three mice were used per treatment group.

Semi-quantitative RT-PCR of mRNA from non-parenchymal liver cells. As shown in Table 2, in all treatment and control groups high levels of HPRT expression were observed. Strikingly, non-parenchymal liver cells did not express TNF–α mRNA nor IL-6

mRNA in any group. IL-2 mRNA expression was enhanced in mice treated with LE-MTPPE/IFN–γ at 12 h and 96 h after end of treatment. Also IL-10 mRNA expression was elevated at 96 h after end of treatment with immunomodulators. However, significant decrease in IL-10 mRNA was observed in mice treated with immunomodulators at 12 h after end of treatment, which decrease was also observed after treatment with PL. IL-4 mRNA expression was decreased at 12 h after end of treatment with LE-MTPPE/IFN–γ, whereas the expression was slightly augmented by administration of PL. Expression of IFN–γwas augmented at 12 h after end of treatment with LE-MTPPE/IFN–γ which was also seen after treatment with PL.

TABLE 2. mRNA expression in 10^6 non-parenchymal liver cells in mice at 12 h and 96 h after treatment with LE-MTPPE/IFN–γ.

		Cytokines tested						
Treatment		TNF–α	IFN–γ	IL-2	IL-4	IL-6	IL-10	HPRT
LE-MTPPE/IFN–γ 12 h		1.0[a] (100)[b]	3.7 (244)	3.7 (163)	2.0 (86)	1.0 (100)	1.0 (67)	5.0 (100)
LE-MTPPE/IFN–γ 96 h		1.0 (100)	5.0 (100)	4.3 (144)	3.3 (118)	1.0 (100)	3.0 (300)	5.0 (104)
PL	12 h	1.0 (100)	3.7 (244)	2.3 (100)	3.6 (154)	1.0 (100)	1.0 (67)	4.5 (90)
PL	96 h	1.0 (100)	5.0 (100)	2.8 (94)	3.5 (124)	1.0 (100)	1.0 (100)	5.0 (104)
PBS	12 h	1.0	1.5	2.3	2.3	1.0	1.5	5.0
PBS	96 h	1.0	5.0	3.0	2.8	1.0	1.0	4.8

Mice were given five dosages of LE-MTPPE/IFN–γ, PL or PBS, after which livers were excised, parenchymal cells removed, mRNA extracted and the cytokine mRNA expression level determined by RT-PCR. Three mice were used per treatment group.
a Expression level of mRNA on gel compared with the positive level (5.0) and the negative level (1.0).
b Expression of mRNA expressed as percentage of the PBS-treated mice is given between hyphen.

Semi-quantitative RT-PCR of mRNA from spleen cells. As shown in Table 3, in all treatment and control groups high levels of HPRT expression were observed. Comparable with the mRNA expression in the liver, also in the spleen no TNF–α or IL-6 mRNA could be detected. IL-2 and IL-4 mRNA expression was augmented 96 h after end of treatment with LE-MTPPE/IFN–γ which was also observed after treatment with PL. The mRNA expression for IL-10 showed striking resemblance with the IL-10 mRNA expression in the liver: increased at 96 h after end of treatment with LE-MTPPE/IFN–γ, but diminished expression at 12 h after end of treatment with the immunomodulator which was also observed after treatment with PL.

DISCUSSION

The results of this study show that treatment of mice with immunomodulators LE-MTPPE/IFN–γ results in increased mRNA expression in liver of IL-2 at 12 h and 96 h after end of treatment. IL-10 mRNA expression in liver and spleen was increased at 96 h after end of treatment with LE-MTPPE/IFN–γ. However, in both liver and spleen a decrease in IL-10 mRNA expression was observed at 12 h after end of treatment, which decrease was

also seen after administration of PL.

Table 3. mRNA expression in 10^6 spleen cells in mice at 12 h and 96 h after treatment with LE-MTPPE/IFN–γ.

		Cytokines tested						
Treatment		TNF–α	IFN–γ	IL-2	IL-4	IL-6	IL-10	HPRT
LE-MTPPE/IFN–γ	12 h	1.0[a] (100)[b]	5.0 (100)	4.0 (100)	5.0 (100)	1.0 (100)	1.3 (53)	5.0 (100)
LE-MTPPE/IFN–γ	96 h	1.0 (100)	5.0 (100)	4.7 (179)	5.0 (136)	1.0 (100)	2.0 (200)	5.0 (100)
PL	12 h	1.0 (100)	4.8 (96)	4.0 (100)	5.0 (100)	1.5 (113)	1.2 (48)	4.8 (96)
PL	96 h	1.0 (100)	5.0 (100)	3.8 (147)	4.5 (123)	1.0 (100)	1.0 (100)	5.0 (100)
PBS	12 h	1.0	5.0	4.0	5.0	1.0	2.5	5.0
PBS	96 h	1.0	5.0	2.6	3.7	1.0	1.0	5.0

Mice were given five dosages of LE-MTPPE/IFN–γ, PL or PBS, after which spleens were excised, mRNA extracted and the cytokine mRNA expression level determined by RT-PCR. Three mice were used per treatment group.
a Expression level of mRNA on gel compared with the positive level (5.0) and the negative level (1.0).
b Expression of mRNA expressed as percentage of the PBS-treated mice is given between hyphen.

Most strikingly no mRNA expression at all was found for TNF–α or IL-6. These results seem in contradiction with the presumed target for immunomodulators. As macrophages have receptors for both MTPPE and IFN–γ, and it was demonstrated that liposomes used in our study are primarily taken up by macrophages, the tissue macrophage is thought to be the primary target for immunomodulators (2-5). Moreover, in vitro studies with macrophages demonstrate an increased TNF–α production after exposure of the cells to MTPPE or IFN–γ (10-12). It was therefore speculated that immunomodulation in vivo would result in pronounced TNF–α production by macrophages. The absence of any TNF–α or IL-6 (which is also closely correlated with an inflammatory response) mRNA expression could indicate that the expression of these cytokines is not induced by LE-MTPPE/IFN–γ or that the time point of measurement (12 h and 96 h after end of treatment) is too late to detect mRNA for these cytokines.

It was previously shown in our laboratory that T-cells play an important role during activation of host defence by LE-MTPPE/IFN–γ (ten Hagen et al; manuscript submitted for publication). This is again confirmed by the observed increase of IL-2 mRNA expression in the present study. Especially endogenous IFN–γ production appeared crucial during this process. IL-10 however, did seem to have a negative effect on host defence activation when administered during immunomodulation. It was also shown that immunomodulation in mice results in predominant IFN–γ producing T-cells, and no IL-4/IL-10 producing T-cells in spleen. From these results it was speculated that especially Th1 cells, NK cells and $CD8^+$ cells (cells known to produce IFN–γ) are important in antimicrobial host defence activation by LE-MTPPE/IFN–γ. A striking observation however is the expression of IL-10 mRNA after immunomodulation in the present study. From these results it becomes clear that IL-10 mRNA expression is initially diminished upon immunomodulation at 12 h after end of treatment, but enhanced at 96 h after end of treatment in both liver and spleen. From these results we speculate that macrophage activation is shut down (by IL-10) somewhere between 12 h and 96 h after end of treatment, because IL-10 has strong

macrophage inhibiting activity (13).

The present results indicate that induction of mRNA for proinflammatory cytokines (e.g TNF–α and IL-6) by immunomodulators is absent already 12 h after end of treatment. IL-2 on the other hand was increased indicating T-cell activity. However, expression of mRNA of cytokines known to inhibit macrophage activity (e.g. IL-10) was enhanced at 96 h after end of treatment. These results indicate that probably macrophage activation and interaction with T-cells, leading to increased antimicrobial host defence activity, is a process which takes place during the first injections of immunomodulator or directly after injection in a transient way.

REFERENCES

1 ten Hagen, T.L.M., van Vianen, W., Bakker-Woudenberg, I.A.J.M. (1995) Modulation of the non-specific antimicrobial resistance of mice by separate or combined liposome-encapsulated muramyl tripeptide phosphatidylethanolamine and interferon-γ towards *Klebsiella pneumoniae* septicemia. *J. Infect. Dis. 171, 385-392.*

2 Melissen, P.M.B., W. van Vianen, P.J.M. Leenen, and I.A.J.M. Bakker-Woudenberg. 1994. Tissue distribution and cellular distribution of liposomes encapsulating muramyltripeptide phosphatidyl ethanolamide. *Biotherapy 7: 71-78.*

3 Fidler, I.J., W.E. Fogler, E.S. Kleinerman, and I. Saiki. 1985. Abrogation of species specificity for activation of tumoricidal properties in macrophages by recombinant mouse or human interferon–γ encapsulated in liposomes. *J. Immunol. 135(6):4289-4296.*

4 Smith, M.R., K. Muegge, J.R. Keller, H-F. Kung, H.A. Young, and S.K. Durum. 1990. Direct evidence for an intracellular role for IFN–γ: microinjection of human IFN–γ induces Ia expression on murine macrophages. *J. Immunol. 144:1777-1782.*

5 Mehta, K., R.L. Juliano, and G. Lopez-Berestein. 1984. Stimulation of macrophage protease secretion via liposomal delivery of muramyl dipeptide derivatives to intracellular sites. *Immunology 51:517-527.*

6 Melissen, P.M.B., W. van Vianen, and I.A.J.M. Bakker-Woudenberg. 1992. Roles of peripheral leukocytes and tissue macrophages in antimicrobial resistance induced by free or liposome-encapsulated muramyl tripeptide phosphatidylethanolamide. *Infect. Immun. 60:2891-2897.*

7 Dijkmans, R., H. Heremans, and A. Billiau. 1987. Heterogeneity of chinese hamster ovaria cell-produced recombinant murine interferon–γ. *J. Biol. Chem. 262:2528-2535.*

8 ten Hagen, T.L.M., van Vianen, W., Bakker-Woudenberg, I.A.J.M. 1996. Isolation and chracterization of murine Kupffer cells and splenic macrophages. *J. Immunol. Methods 193;81-91.*

9 Chomczinski, P. Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorofrom extraction. *Anal. Biochem.* 162:156-159.

10 O'Reilly, T., and Zak, O. 1991. Enhancement of the effectiveness of antimicrobial therapy by muramyl pepetide immunomodulators. *Clin. Infect. Dis. 14:1100-1109.*

11 Langermans, J.A.M., van der Hulst, M.E.B., Nibbering, P.H., Hiemstra, P.S., Fransen, L., and van Furth, R. 1992. IFN–γ-Induced L-Arginine Dependent Toxoplasmastatic Activity in Murine Peritoneal Macrophages is Mediated by Endogenous Tumor Necrosis Factor–α. *J. Immunol. 48:568-574.*

12 Neta, R., Sayer, T.J., and Oppenheim, J.J. 1992. Relationship of TNF to interleukins. *Immunology Series 56:499-566.*

13 Mosmann, T.R. 1994. Properties and Functions of Interleukin-10. *Adv. Immunol. 56:1-26.*

CHAPTER SIX

Enhancement of Nonspecific Resistance by Liposome-encapsulated Immunomodulators does not Affect Skin Graft Rejection in Mice.

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ABSTRACT

Administration of liposome-encapsulated immunomodulating agents muramyl tripeptide phosphatidyl ethanolamine (LE-MTPPE) or interferon-γ (LE-IFN–γ), or co-encapsulated MTPPE and IFN–γ (LE-MTPPE/IFN–γ) resulted in a dramatic increase of the nonspecific antimicrobial resistance in mice as shown before. This kind of treatment is especially of use in immunocompromised hosts who are prone to severe infections. Application of these immunomodulators might protect these patients, e.g. transplant recipients, from opportunistic infections. However, accelerated rejection of the graft, resulting from augmentation of the antimicrobial defense in a nonspecific way, has to be avoided.

In this study the effect of treatment with LE- MTPPE, LE- IFN–γ, or LE-MTPPE/IFN–γ on skin graft rejection in mice was investigated. It was found that prophylactic treatment of skin grafted mice with immunomodulating formulations did not influence rejection of the graft. Moreover, in T cell-depleted mice, which showed a prolonged graft survival compared with immunocompetent recipients, the administration of immunomodulators did not change the survival time of the grafts compared with T cell-depleted mice that did not receive immunomodulators. The results clearly show that in this experimental setting application of the antimicrobial resistance-enhancing formulations (LE-MTPPE, LE-IFN–γ, and LE-MTPPE/IFN–γ is allowed in graft bearing recipients, without influencing graft survival.

INTRODUCTION

Activation of the host defense system in a nonspecific way proved beneficial in several infection models [1,2,3,4,5,6]. Treatment with liposome-encapsulated immunomodulating agent muramyl tripeptide phosphatidyl ethanolamine (LE-MTPPE) in combination with interferon–γ (IFN–γ) resulted in strong potentiation of the antimicrobial host resistance [7]. This kind of treatment might be of special use in the immunodeficient host, who is prone to severe infections. Therefore one group of interest might be transplant recipients, who receive immunosuppressive agents to prevent graft rejection. A major risk factor in this patient group might be that augmentation of the host defense with the above mentioned immunomodulators, to prevent infections, at the same time might result in acceleration of graft rejection.

Administration of OKT3, a mouse anti-CD3 mAb to patients has been shown to be effective in prevention of organ allograft rejection [8,9,10], as could be achieved through T-cell depletion in mice [11]. Because these agents and other established immunosuppressive agents are rather nonspecific, transplant recipients experience an increased incidence of infection [12,13]. Administration of immunomodulating agents seems therefore of great value in this group of patients.

In the present study the effect of LE-MTPPE, LE-IFN–γ or both LE-MTPPE/IFN–γ on graft survival was studied in immunocompetent mice and mice treated with anti-CD3 mAb to examine whether potentiation of the nonspecific host defence would change the survival time of the skin transplant in mice.

MATERIALS AND METHODS

Animals. Specified pathogen free, 11- to 13-week old female C57Bl/Ka BL-1 (H-2^b) mice were obtained from ITRI-TNO, Rijswijk, The Netherlands. B6.C-H-2^{bm12} (H-2^{bm12}) mice were bred at the Department of Immunology, Erasmus University Rotterdam, The Netherlands.

Reagents. LE-MTPPE and placebo liposomes (PL) were kindly provided by Ciba Geigy Ltd. (Basel, Switzerland). Recombinant mouse IFN–γ (rMuIFN–γ) was provided by Prof. A. Billiau (Rega Institute, Leuven, Belgium). rMuIFN–γ was derived from the supernatant fluid of Mick cells, a CHO cell line that carries and expresses an amplified murine IFN–γ cDNA [14]. This interferon was purified by affinity chromatography on DB1-mAb. Liposomes containing MTPPE or IFN–γ and liposomes containing MTPPE as well as IFN–γ were prepared as described previously [7].

T cell depletion. Mice were rendered T cell deficient by injecting 100 μg of 17A2 (rat IgG2b mAb against CD3 [15,16] intraperitoneally, 8 days before transplantation. This dosage was shown to result in profound T cell depletion for at least 2 weeks and to induce a significant prolongation of skin graft survival as compared with immunocompetent controls [11].

Immunomodulation. At 7 days before transplantation, intravenous treatment with LE-MTPPE (25 μg), LE-IFN–γ (7500 U) or LE-MTPPE/IFN–γ (25 μg MTPPE and 7500 U IFN–γ) was started and continued every other day. The last two dosages were administered at 24 hr and 12 hr before transplantation as described previously [7]. Mice treated with PL received 6.25 mg of lipid per dosage, which equals the amount of lipid administered in the other groups. Every treatment group had his own control group treated with PL. One group of mice did not receive liposomes and served as controls for the PL treated mice.

Transplantation. Tail skin of bm12 donors was grafted to the back of C57Bl/Ka BL-1 recipients using a modified method of Billingham et al, as was described previously [11,17]. Grafts were considered rejected when no viable donor skin was detectable macroscopically.

Statistical analysis: Statistical evaluation of differences in survival curves of the grafts between different groups of mice was performed by using the log rank test.

RESULTS

Effect of treatment with PL on skin graft rejection in mice. From the skin graft survival curves it became clear that treatment of mice with PL had no effect on transplant rejection compared with treatment of mice that did not receive liposomes, both in immunocompetent and in anti-CD3 immunocompromised mice (figure 1).

Effect of treatment with LE-MTPPE on skin graft rejection in mice. The anti-CD3 treatment resulted in a significant increase in transplant mean survival time from 16 to 70 days. Treatment of immunocompetent mice with LE-MTPPE had no effect on the transplant survival time as compared with survival time for mice treated with PL (figure 1). Also, prolongation of skin graft survival through T cell depletion was not influenced by treatment with LE-MTPPE.

Effect of treatment with LE-IFN–γ on skin graft rejection in mice. Treatment of immunocompetent mice with LE-IFN–γ did not influence graft rejection compared with PL-treated mice (figure 2). Prolongation of skin graft survival through T-cell depletion was also not changed by the administration of LE-IFN–γ.

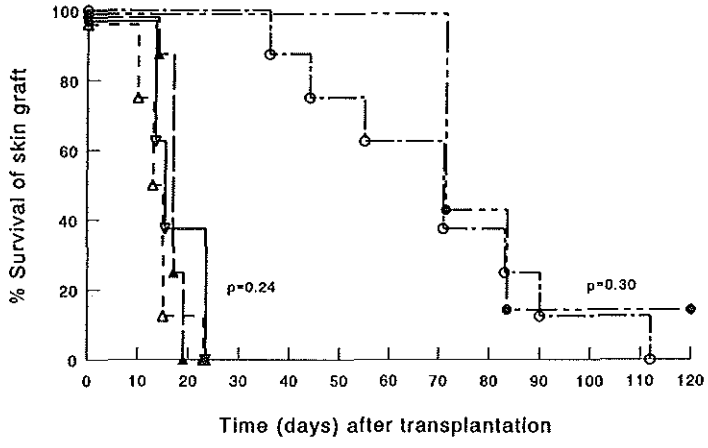

Figure 1. Skin graft survival in immunocompetent mice treated with LE-MTPPE (▵) or PL (▿), and in mice depleted of T cells treated with LE-MTPPE (○) or PL (●). Skin graft survival in control mice that did not receive liposomes (▲). Eight mice per group were used.

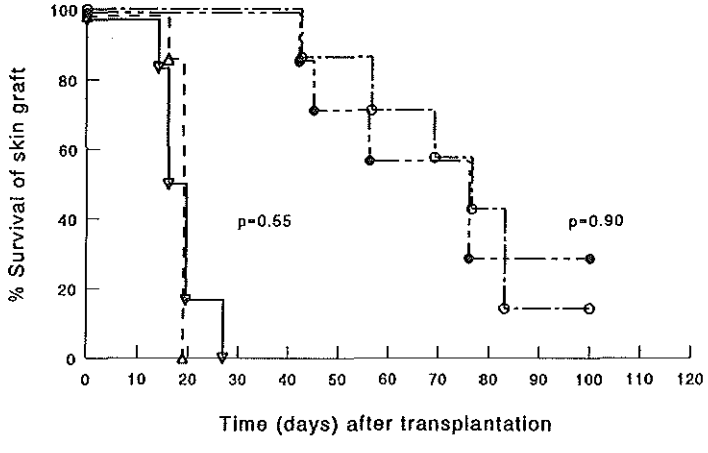

Figure 2. Skin graft survival in immunocompetent mice treated with LE-IFN-g (▵) or PL (▿), and in mice depleted of T cells treated with LE-IFN-g (○) or PL (●). Seven mice per group were used.

Effect of treatment with co-encapsulated MTPPE plus IFN-γ on skin graft rejection in mice. Administration of LE-MTPPE/IFN-γ also had no effect on skin graft survival time compared with PL-treated mice, in both the immunocompetent as well as the T-cell depleted mice (figure 3).

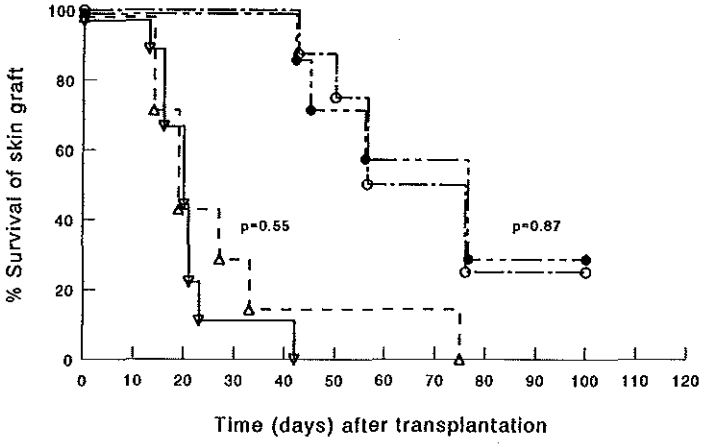

Figure 3. Skin graft survival in immunocompetent mice treated with LE-MTPPE/IFN-γ (△) or PL (▽), and in mice depleted of T-cells treated with LE-MTPPE/IFN-γ (○) or PL (●). Seven mice per group were used.

DISCUSSION

Suppression of the immune system is necessary for prevention of graft rejection. As demonstrated in mice before, the application of T-cell depleting antibodies is very effective in prolongation of skin graft survival time [11]. Unfortunately, the application of immunosuppressive agents renders the transplant recipient vulnerable to opportunistic infections [12,13]. Therefore, patient survival is not only threatened by rejection of the graft, but by infections as well. These infections are complicated and life threatening, because failure of antibiotic treatment of infections in these patients occurs frequently [18,19,20]. Stimulation of the antimicrobial resistance preferably in a nonspecific way might support the antibiotic treatment of infections. In previous studies we observed that prophylactic treatment of mice with LE-MTPPE or LE-IFN-γ could protect mice from bacterial infections. Administration of co-encapsulated MTPPE and IFN-γ even resulted in 100% survival of mice with a normally lethal septicemia with *Klebsiella pneumoniae* [7]. Treatment with co-encapsulated MTPPE and IFN-γ also proved effective in mice with an intracellular Leishmania infection as described by Hockertz et al [21]. Even in T cell deficient mice with *K. pneumoniae* septicemia, significant stimulation of the nonspecific resistance by LE-MTPPE, LE-IFN-γ or LE-MTPPE/IFN-γ was demonstrated [22, ten Hagen et al., manuscript in preparation].

Although treatment of infection in mice with liposome-encapsulated immunomodulators has been shown to be effective, the application of this treatment might be restricted in transplant recipients. Transplant patients treated with immunosuppressive agents are also prone to infection. However, it is anticipated that stimulation of the nonspecific antimicrobial resistance at the same time will result in acceleration of graft rejection, if the immunomodulators stimulate macrophages that activate graft-reactive T

cells. The activation of the immune system might even counteract the immunosuppression induced to protect the graft from rejection.

The present study shows that administration of the potent immunomodulators LE-MTPPE and LE-IFN–γ to immunocompetent mice did not influence the rejection of skin grafts in mice. Moreover, administration of co-encapsulated MTPPE plus IFN–γ, in our hands the most potent formulation to increase antimicrobial resistance, did not accelerate graft rejection.

It has been observed that T cell depletion of mice by anti-CD3 mAb before transplantation resulted in a significantly prolonged survival time of the skin graft [11]. The present study shows that skin graft survival in T cell-depleted mice, treated with liposomal immunomodulator formulations, was not changed compared with the placebo treated controls. These results indicate that administration of potent antimicrobial resistance-activating agents is permitted in skin grafted mice. Moreover, treatment of T-cell depleted mice with liposomal immunomodulators also did not affect skin graft rejection. Actually, activation of the nonspecific resistance by these formulations was expected to accelerate graft rejection. Failure of the immunomodulators to accelerate graft rejection might be explained by the observation that within 30 min. after intravenous injection already 55% of the liposomes are located in the liver, spleen and lung [23]. It is most likely that these liposomes are taken up by the cells of the mononuclear phagocyte system (MPS), which results in rapid clearance of the liposomes. Only few or no liposomes will reach the graft. As a result, local activation of host resistance is not expected.

Biodistribution of the liposomes is dependent upon various factors, such as charge, size and lipid composition [24]. The type of liposomes used in this study are preferentially taken up by the cells of the MPS, particularly in the liver and spleen. However, the liposomes used in the present study are very heterogeneous in size. The small liposomes in this formulation are taken up less easily by cells of the MPS, and are probably able to leave the circulation and extravasate at the site of the transplant. As a result, clearance of liposomes by the MPS is not very likely to account for the failure of the immunomodulators to accelerate transplant rejection. Moreover, stimulation of host resistance by liposomal immunomodulators not only is through direct activation of the cells of the MPS, but also is dependent upon second signals from other cells [7]. Administration of liposomal MTPPE or co-encapsulated MTPPE plus IFN–γ resulted in stimulation of the macrophage, but recruitment of granulocytes was also observed [25]. Studies of the mechanisms behind host resistance stimulation by LE-MTPPE/IFN–γ revealed that next to granulocytes, other cells (macrophages and erythroblasts) also increased in number [ten Hagen et al., manuscript in preparation]. The dramatic increase of cells results from systemic production of colony stimulating factors and other cytokines by macrophages or cells stimulated by macrophages [26,27]. These observations indicate that activation of the macrophage with liposome-encapsulated immunomodulators results in a wide spectrum of activities, other then direct macrophage activation. Localization of the liposome away from the graft is therefore not a likely explanation for failure of accelerated graft rejection.

We observed that activation of host resistance with liposomal immunomodulators augments antimicrobial resistance, but is without effect on skin graft rejection. This might suggest that activation of resistance results in increased activity of the MPS, without activation of the specific immune system. However, previous studies demonstrated that

T cells play a significant role in stimulation of the host resistance by these agents [22, ten Hagen et al., manuscript in preparation]. Activation of the resistance with LE-MTPPE, LE-IFN–γ or co-encapsulated LE-MTPPE/IFN–γ does not include antigens specific for the transplant. Therefore, activation of T cells directed to the transplant, or selection of these cells does not occur. An explanation might be that the T cells involved in activation of the nonspecific resistance are not the same as the T cells involved in the specific immune reaction necessary for transplant rejection. This is supported by observations that effective treatment with liposomal immunomodulators is greatly reduced when T cells are not present, whereas effective treatment in competent mice did not result in acceleration of graft rejection. We also believe that, in a syngenic transplant model no acceleration of graft rejection will occur. In graft rejection the specific resistance is believed to play a major role [28]. Absence of accelerated graft rejection would therefore be explained by the ability of the immunomodulators to stimulate resistance in a nonspecific way, independent of the following challenge.

REFERENCES

1 Badaro R, Johnson Jr WD. The role of interferon-γ in the treatment of visceral and diffuse cutaneous leishmaniasis. J Infect Dis 1993; 167(Suppl 1): S13.

2 Czarniecki CW, Sonnenfeld G. Interferon-gamma and resistance to bacterial infections. APMIS 1993; 101: 1.

3 Fraser-Smith EB, Eppstein DE, Larsen MA, Matthews TR. Protective effect of a muramyl dipeptide analog encapsulated in or mixed with liposomes against *Candida albicans* infection. Infect Immun 1983; 39(1): 172.

4 Koff WC, Showalter SD, Hampar B, Fidler IJ. Protection of mice against fatal herpes simplex type 2 infection by liposomes containing muramyl tripeptide. Science 1985; 228: 495.

5 Melissen PMB, Van Vianen W, Bidjai O, Van Marion M, Bakker-Woudenberg IAJM. Free versus liposome-encapsulated muramyl tripeptide phosphatidylethanolamide (MTPPE) and interferon–γ (IFN–γ) in experimental infection with *Listeria monocytogenes*. Biotherapy1993; 6: 113.

6 O'Reilly T, Zak O. Enhancement of the effectiveness of antimicrobial therapy by muramyl peptide immunomodulators. Clin Infect Dis 1992; 14: 1100.

7 ten Hagen TLM, van Vianen W Bakker-Woudenberg, IAJM. Modulation of the Non-Specific Antimicrobial Resistance of Mice by Separate or Combined Liposome-encapsulated Muramyl Tripeptide Phosphatidylethanolamine and Interferon-γ towards *Klebsiella pneumoniae* Septicemia. J Infect Dis 1995; 171: 385.

8 Norman DJ, L Kahana, Stuart, FPJ, et al. A randomized clinical trail of induction therapy with OKT3 in kidney tranpslantation. Transplantation 1993; 55(1): 44.

9 Ortho Multicenter Transplant Study Group. A randomized clinical trail of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. N Engl J Med 1985; 313(6): 337.

10 Parlevliet KJ, Schellekens PTA. Monoclonal antibodies in renal transplantation: a review. Transplant Int 1992; 5: 234.

11 Vossen ACTM, Knulst AC, Tibbe GJM, van Oudenaaren A, Beart MRM, Benner R, Savelkoul HFJ. Suppression of skin allograft rejection in mice by anti-CD3 monoclonal antibodies without cytokine-related side-effects. Transplantation 1994; 58(2): 257.

12 Hesse UJ, Wienand P, Baldamus C, Pollok M, Pichlmaier H. The risk of Infection following OKT3 and antilymphocyte globulin treatment for renal transplant rejection: results of a single center prospectively randomized trial. Transplant Int 1992; 5(suppl 1): S440.

13 Thistlethwaite JR, Stuart JK, Mayes JT, Gaber AO, Woodle S, Buckingham MR, Stuart FP. Monitoring and complications of monoclonal therapy: Complications and monitoring of OKT3 therapy. Am J Kidney Dis 1988; 11(2): 112.

14 Dijkmans R, Volckaert G, Van Damme J, De Ley M, Billiau A, De Somer P. Molecular cloning of murine interferon gamma (MuIFN–γ) cDNA and its expression in heterologous mammalian cells. J Interferon Res 1985; 5: 511.

15 Miescher GC, Schreyer M, MacDonald HR. Production and Characterization of a rat monoclonal antibody against the murine CD3 molecular complex. Immunol Lett 1989; 23: 113.
16 Savelkoul HFJ, Vossen ACTM, Breedland EG, Tibbe GJM. Semi-preperative purification and validation of monoclonal antibodies for immunotherapy in mice. J Immunol Methods 1994; 172: 33.
17 Billingham RE, Medawar PB. The technique of free skin grafting in mammals. British Empire Cancer Campaign Research Fellow 1951; 385.
18 Soave R, Sepkowitz KA. The immunologic compromised host. In: Reese RE, Betts RF eds. A practical approach to infectious diseases. London: Little, Brown and Company, 1991: 566.
19 Bone R. Gram-negative sepsis: a dilemma of modern medicine. Clin Microbiol Reviews 1993; 6(1):57.
20 Umsawasdi T, Middleman EA, Luna M, Bodey G. Klebsiella bacteremia in cancer patients. Am J Med Sci 1973; 265: 474.
21 Hockertz S,Franke G , Paulini I, Lohmann-Matthes M-L. Immunotherapy of murine visceral leishmaniasis with murine recombinant interferon-γ and MTP-PE encapsulated in liposomes. J Interferon Res 1991; 11(3): 177.
22 ten Hagen TLM, van Vianen, W Straathof EAT, Bakker-Woudenberg IAJM. Effect of liposome-encapsulated muramyl tripeptide phosphatidylethanolamine and interferon gamma on *Klebsiella pneumoniae* infection in T-cell-deficient mice and in vitro. In: Masihi KN, ed. Immunotherapy in infections Marcel Dekker Inc, New York 1994; 225.
23 Melissen PMB, van Vianen W, Leenen PJM, Bakker-Woudenberg IAJM. Tissue distribution and cellular distribution of liposomes encapsulating muramyl tripeptide phosphatidyl ethanolamide. Biotherapy 1994; 7: 71.
24 Gregoriadis G. Overview of liposomes. J Antimicrob. Chemother 1991; 28(suppl) B: 39.
25 Melissen PMB, van Vianen W, Bakker-Woudenberg IAJM. Roles of peripheral leukocytes and tissue macrophages in antimicrobial resistance induced by free or liposome-encapsulated muramyl tripeptide phosphatidyl ethanolamide. Infect Immun 1992; 60(11): 2891.
26 Fedoročko P. Liposomal muramyl tripeptide phosphatidylethanolamine (MTP-PE) promotes haemopoietic recovery in irradiated mouse. Int J Radiat Biol 1994; 65(4):465.
27 Hadden JW. Immunostimulants. Imunol Today 1993; 14(6): 275.
28 Rosenberg A, Mizuochi T, Sharrow S, Singer A. Phenotype, specificity and function of T cell subsets and T cell interactions involved in skin allograft rejection. J Exp Med 1987; 165: 1296.

CHAPTER SEVEN

Isolation and Characterization of Murine Kupffer Cells and Splenic Macrophages.

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ABSTRACT

A method is described using counterflow centrifugation elutriation to isolate macrophages from murine liver and spleen. In this study three, size fractionated, macrophage populations were collected. Isolation resulted in a high yield of pure Kupffer cells (total of $10X10^6$ /g liver) and enrichment of splenic macrophages to 20%. In addition to standard methods such as non-specific esterase staining, the isolated macrophages were also characterized by flow cytometry using specific monoclonal antibodies. In addition, a rapid flow cytometry method was introduced to determine the percentage of macrophages bases on autofluorescence. A strong correlation was found between the percentages of macrophages found by non-specific esterase staining and autofluorescence. Functional tests revealed differences between the isolated macrophages in terms of TNF-α production, oxygen metabolism and the production of nitric oxide. However, no significant differences in phagocytic activity was observed between the fractions. After two weeks of culture without the addition of antibiotics the cells still exhibited the above mentioned functions.

INTRODUCTION

Isolation of Kupffer cells (KC) from mice by counterflow centrifugation elutriation (CCE) was described in 1977 by Knook and Sleyster (1977). More recently the purification of KC from mice, using the same technique has been reported by other workers (Janousek et al., 1993). Macrophages isolated under these conditions prompted the examination of functional characteristics in vitro, and provide excellent starting material. Moreover, several methods have been described using CCE for the isolation of KC from rat liver. Some of these methods are widely used and provide researchers with a fairly pure, though heterogeneous population of KC.

The isolation of splenic macrophages is far more difficult. The spleen possess different types of macrophages: the marginal zone macrophages, macrophages of the red pulp, the peri-arteriolar sheet, and the marginal metallophils, all of which possess different characteristics (Humphrey and Grennan 1981). Relatively few methods have been described for the isolation of splenic macrophages (Buckley et al., 1984; Ogle et al., 1994; Thompson et al., 1983). Due to the size of the organ, and the percentage of macrophages in the spleen, the collection of reasonable numbers of macrophages from the murine spleen is not easy.

It is clear that the spleen comprises different types of macrophages. However, the findings of others suggest that in the rat also the KC population is heterogeneous (Hoedemakers et al., 1993). Our own studies on murine liver and spleen by flow cytometry have indicated different activities of macrophages in these organs, which is in accordance with the findings of other groups working with rat liver (Daemen et al., 1989,Daemen et al., 1991, Hardonk et al., 1989, Itoh et al., 1992).

Here we describe a method using CCE for the isolation of macrophages from mouse liver and spleen. The tissue macrophages were isolated under sterile conditions using an autoclavable elutriation rotor, which facilitates culture without the use of antibiotics. The

cells were characterized by flow cytometry using monoclonal antibodies which differentiate between the macrophages and other cells, and between the different types of macrophages. Autofluorescence was used as an indicator of the percentage of macrophages in the cell populations, and showed a high correlation with the standard non-specific esterase method. In addition, the cells were cultured under more physiological non-adherent conditions, without the addition of antibiotics.

MATERIALS AND METHODS

Animals. Specified pathogen free, 13 to 15 week old male C57Bl/Ka mice were used (ITRI-TNO, Rijswijk, The Netherlands). The experimental design and animal care were performed according to institutional guidelines.

KC isolation. Mice were anaesthetized i.p. with Nembutal (Sanofi, Paris, France). A ventral midline incision exposed the peritoneal cavity, and a sterile 24-gauge Teflon cannula (introcan-w, 24G¾, Braun Melsungen, Melsungen, Germany) was inserted into the vena porta. The liver was preperfused *in situ* with calcium free Hank's buffered salt solution (Ca^{2+}-free HBSS low endotoxin) (HyClone Laboratories, Logan, UT, USA) at pH 7.4 and 30°C, allowing the blood to flow from an incision in the vena cava inferior. After 2 min the wash solution was replaced by collagenase A solution (0.163 U/ml in DMEM high glucose)(Boehringer Mannheim, Mannheim, Germany), and the liver was perfused at a flow rate of 5 ml/min for 30 s at 35°C. After perfusion the liver was removed from the mouse and gently dispersed. Liver suspensions from eight mice were pooled and incubated under continuous agitation at 35°C for 10 min in 40 ml collagenase solution as was used for the perfusion in 50 ml polypropylene tubes. Hereafter all experiments were performed at 4°C. The liver cell suspension was filtered through a nylon gauze (212 μm) and taken up in 50 ml HBSS containing 0.3% BSA (fraction V, low endotoxin, Sigma, St Louis, MO, USA) and 2 μg/ml DNase (low endotoxin, sterile, Boehringer Mannheim, Mannheim, Germany), and centrifuged at 400 X g for 15 min. This buffer is subsequently referred to as working buffer (WB). The pellet was taken up in 15.6 ml WB and mixed with 21 ml NycoPrep (low endotoxin, sterile Nycodenz solution with a density of 1.150 g/ml and osmolarity of 290 mosM, Nycomed Pharma, Oslo, Norway). The mixtures were transferred to three 15 ml polypropylene tubes, and 1 ml WB was layered on top of the mixture. Red blood cells and debris was sedimented by centrifugation at 1500 X g for 15 min. The cells in the interphase, which were mostly sinusoidal cells, were suspended in 50 ml WB and washed twice to remove Nycodenz by centrifugation at 400 X g for 15 min. The pellet was taken up in 5 ml WB and the cells further separated into various fractions by CCE using the autoclavable Beckman JE-5.0 elutriator rotor supplied with a Sanderson chamber (Beckman Instruments, Palo Alto, CA, USA). The cell suspension was introduced into the elutriator at a flow rate of 12 ml WB per min, and the elutriator speed set to 3200 rpm. DNase was added to flow-buffer (2 mg/l in HBSS/0.3%BSA) to prevent cell clumping in the elutriator chamber. Fractions of 100 ml were collected at flow rates of 12, 18, 26, 32 and 50 ml/min.

Isolation of splenic macrophages. Spleens were excised from anaesthetized mice (see KC isolation) and spliced once with forceps. Organs from 15 mice were incubated under continuous agitation for 20 min at 35°C in 20 ml collagenase A solution (0.163 U/ml in HBSS). Thereafter the digest was gently pushed through a nylon gauze (88 μm), and the cell suspension collected in 50 ml WB containing 0.5 IU heparin. The cells were pelleted by centrifugation at 400 X g for 15 min, resuspended in 5.5 ml WB and mixed with 7 ml Nycodenz. To prevent the cells in the interface from dehydration 1 ml WB was layered on top. The gradient was centrifuged for 15 min at 1500 X g. The interface cells were transferred to a 50 ml polypropylene tube containing WB and washed twice by centrifugation at 400 g for 15 min. The pelleted cells were resuspended in 5 ml WB and

introduced into the elutriator at a flow rate of 12 ml WB per min, and the rotor speed set at 3200 rpm. Fractions between 100 and 400 ml were collected at flow rates of 12, 18, 24, 36 and 50 ml/min.

Culture of isolated KC and splenic macrophages. Isolated macrophages from fraction 3, 4, and 5 of the liver and spleen were brought to a density of $1.0X10^6$ cells per ml in DMEM high glucose culture medium (Gibco, Life Technologies, Gaithersburg, MD), supplemented with 15% FCS (Gibco). The macrophages could be cultured in 5% CO_2 for up to two weeks at 37°C and in 96 well plates (MatTek corp, Ashland, Ma, USA), using 150.000 cells per well, with continuous shaking at 110 rpm, or in plastic 96 well Falcon culture plates (Becton Dickinson, San Jose, CA).

Characterization of isolated cell populations. Flow cytrometric analysis was performed using of rat anti-mouse monoclonal antibodies (mAb) as hybridoma culture supernatants. F4/80 (murine Mϕ)(Austyn and Gordon, 1981), MOMA-2 (murine Mϕ)(Kraal et al., 1987), RA3-6B2 (B-cells)(Coffman, 1982), KT3 (T-cells)(Tomonari, 1988), 30G12 (general leukocyte marker)(Ledbetter and Herzenberg, 1979), and Y3 a non-reacting negative control. All hybridoma supernatants were kindly provided by Dr P. Leenen and J. Voerman (Department of Immunology, Erasmus University, Rotterdam, Netherlands). 25 µl cell suspension was mixed with 25 µl hybridoma supernatant and incubated at room temperature for 10 min. Cell suspensions were washed three times with PBS containing 5% FCS and 20 mM sodium azide. Thereafter cells were incubated with an FITC-conjugated rabbit anti-rat antibody, $(F(ab')_2$ fragment). Free conjugate was removed by washing as described above. The cell suspensions were examined on a FACScan and evaluated with Consort 30 software (Becton Dickinson, San Jose, CA)

Determination of cell viability. Cells were incubated after isolation with 50 µg/ml propidium iodide (PI) (Sigma, St Louis, MO, USA). Dead cells take up PI which intercalates with the DNA resulting in red fluorescent cells. The percentage of dead cells was determined by flow cytometry.

Autofluorescence (AF) as a quantitative marker of macrophage percentages in cell populations. Macrophages are cells with a pronounced autofluorescent activity (Thorell, 1981). The percentage of autofluorescent cells (AF^+) was evaluated by flow cytometry in the green emission range (Fl1) (excitation 488 nm, emission 520 nm).

Nonspecific esterase (NSE) activity of the purified cell fractions. The purity of the cell fractions was verified on cytospin preparations after staining for NSE activity (Stroben, 1994). Briefly, cells were fixed with ice-cold formalin-acetone and incubated with pararosaniline/α-naphthyl butyrate. Cells were then counterstained with Gills hematoxylin and examined microscopically.

Quantitation of production of reactive oxygen (ROI) and nitrogen (RNI) intermediates. Oxygen metabolism was examined by chemiluminescence 4 h after isolation. Briefly, $1X10^6$ cells in 1 ml were mixed with 100 µl $5X10^{-3}$ M luminol (Sigma, St. Louis, MO, USA), and triggered with 100 µl $5X10^{-5}$ M phorbol myristate acetate (PMA, Sigma, St. Louis, MO, USA). Chemiluminescence was measured using an AutoLumat LB953 (EG&G Berthold, Wildbad, Germany). Nitrite production was measured using Griess reagents as reported by Stuehr and Nathan (1989) 6 days after isolation. Briefly, $1.5X10^5$ macrophages in 150 µl DMEM (without phenol red) supplemented with 10% FCS, were exposed to heat killed *Klebsiella pneumoniae* for 0, 1, 2 or 4 h, and then 150 µl of Griess reagents were added. Absorption at 570 nm was determined and compared with a standard curve prepared at the same time using known concentrations of sodium nitrite.

Assessment of TNF-α production by KC. After 6 days of culture KC were exposed to heat killed *Klebsiella pneumoniae* using a ratio of 15 bacteria per macrophage for 4 h. TNF-α levels were determined in the culture medium using the WEHI 164 bioassay (Espevik and Nissen-Meyer, 1986).

Phagocytic assay. The microorganism used for phagocytosis was *Candida albicans*, grown for 24 h at 37°C in Sabouraud maltose broth (Oxoid, Basingstoke, England), washed three times with PBS and preserved on ice. KC fractions were cultured in 96 well plates at 200 000 cells per well for 6 days in 200 µl DMEM supplemented with 15% FCS. After that cells were incubated with *C. albicans* using a ratio 50 microorganisms per macrophage. Non phagocytosed organisms were

removed by washing the monolayers with PBS, and phagocytosis was examined microscopically after Diff-Quick staining.

RESULTS

Characterization of isolated KC. Directly after separation by CCE, cell-fractions were examined with respect to total cell number and the percentages of different cell types. Fraction 1, collected at a flow rate of 12 ml/min, only contained debris and no cells are present in this fraction. Fraction 2 contained large numbers of endothelial cells and KC. Routinely around $80X10^6$ KC were recovered in Fraction 3 and around $20X10^6$ KC were recovered in Fraction 4, whereas $10X10^6$ KC were collected in Fraction 5 at 50 ml/min. A total of $10X10^7$ KC were collected from 8 mice. The viability of these cells, as assessed by propidium iodide exclusion after isolation, appeared to be between 85 and 95%.

Table 1 Characteristics of Kupffer cells isolated from murine liver by counterflow elutriation centrifugation (CCE).

	Kupffer cell fraction number			
	2	3	4	5
fraction size (ml)[a]	100	100	100	100
number of cells ($X10^6$)	57.3±17.2	79.9±22.3	21.4±6.4	10.5±3.3
% positive cells[b]				
NSE[c]	41.3±3.4	80.9±1.2	>92.5	>95
DQ	17.9±1.3	86.7±2.1	>92.5	>95
F4/80	20.9±8.1	50.2±8.1	56.1±5.7	47.3±3.7
MOMA-2	32.1±4.2	61.7±4.7	68.2±4.8	58.4±7.7
AF	51.9±9.7	80.0±1.0	88.9±4.4	90.5±3.6

a fraction collected from the CCE.
b percentage of positive cells were determined for nonspecific esterase activity and after Diff Quick staining on cell cytospins on slides by counting 400 cells. Percentage of F4/80 or MOMA-2 positive cells was determined by flow cytometry.
c NSE: nonspecific esterase, DQ: Diff Quick, F4/80 and MOMA-2: macrophage specific monoclonal antibodies, AF: autofluorescence.
The data respresent the mean ± 1 SD of at least six experiments.

As shown Table 1, an increasing percentage of macrophages detected by NSE staining were collected by increasing flow rates: NSE positive cells increased from approximately 30% in the unseparated fraction to above 95% in Fraction 5. These results were confirmed by Diff-Quick staining, which clearly showed the presence of the majority of the macrophages in the last three fractions, recognizable by their asymmetric nucleus, vacuolized cytoplasm and membrane ruffling (Fig. 1).

Flow cytometry was also used to quantify KC. Whereas only 50.2, 56.1 and 47.3% of the cells in Fractions 3, 4 and 5 respectively were found to be positive for F4/80 , MOMA-2 recognized 61.7, 68.2 and 58.4% of the cells in the Fractions 3, 4 and 5 when FITC conjugated antibodies were used (Table 1). Using AF to evaluate the macrophage content of the fractions, 51.9% of the cells appeared to be positive in Fraction 2, whereas

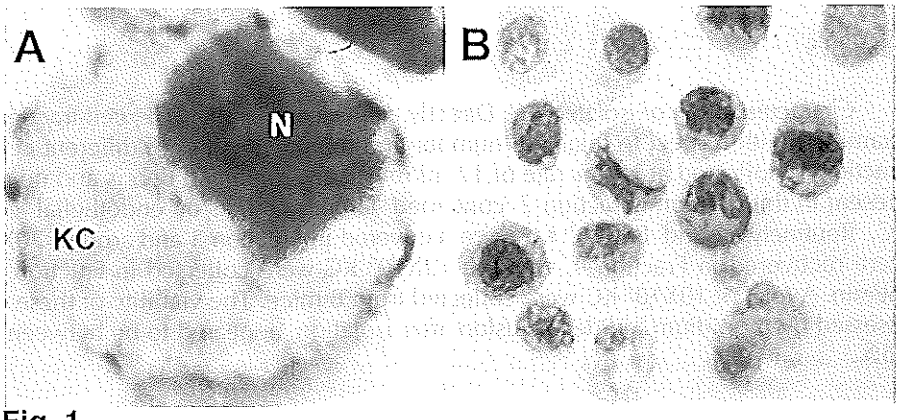

Fig. 1

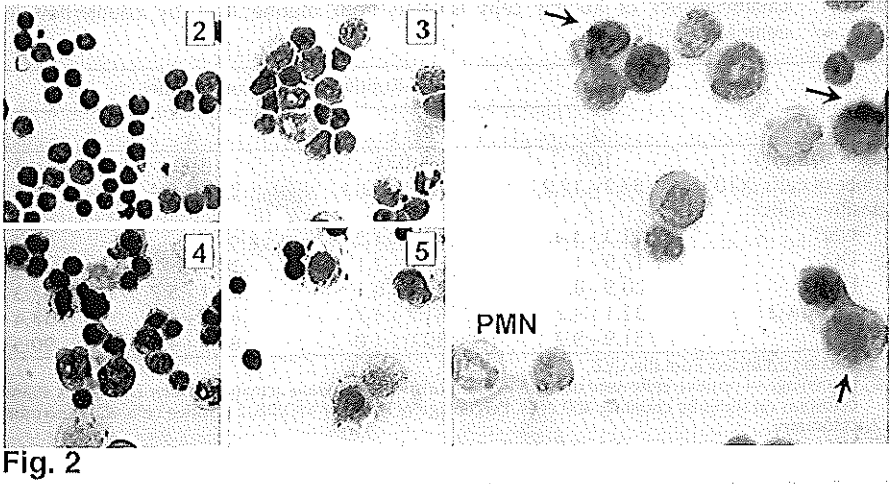

Fig. 2

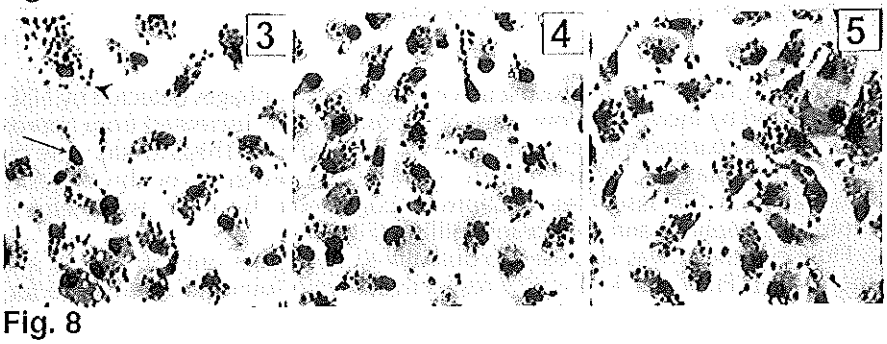

Fig. 8

Fraction 3, 4 and 5 contained 80.0, 88.9 and 90.5% AF^+ cells respectively.

Characterization of isolated splenic macrophages. Fraction 1, collected at 12 ml/min did not contain any cells. Splenic macrophages were present in Fractions 3, 4 and 5, as confirmed by cytospin preparations followed by Diff-Quick or NSE staining (Fig. 2). Less then 1% of the unseparated cells were found to be NSE positive. Flow cytrometric analysis revealed that 11.4, 21.8 and 21.9% of the cells were positive for F4/80 whereas 6.4, 19.6 and 19.4% of the cells stained with MOMA-2 in Fractions 3, 4 and 5 respectively (Table 2). The percentages of AF^+ cells determined by flow cytometry in spleen Fractions 2, 3, 4 and 5 were <1, 4.9, 21.8 and 21.6% respectively.

Table 2 Characteristics of macrophages isolated from murine spleen by counterflow elutriation centrifugation (CCE).

	spleen cell fraction number			
	2	3	4	5
fraction size (ml)[a]	100	400	200	200
number of cells ($X10^6$)	952±188	174±83.6	78.7±34.7	17.5±5.0
% positive cells[b]				
NSE[c]	<1	4.0±0.3	17.6±1.8	18.3±1.9
DQ	<1	10.3±1.1	11.5±1.3	14.0±1.5
F4/80	2.2±0.4	11.4±4.7	21.8±4.5	21.9±6.4
MOMA-2	1.8±0.4	6.4±1.4	19.6±1.3	19.4±6.3
AF	0.9±0.6	4.9±3.3	12.8±6.51	4.3±3.7
KT3	25.7±5.8	16.0±0.2	8.6±0.12	1.5±0.4
RA3-6B2	46.4±6.4	41.9±0.1	33.3±0.4	34.7±0.7
RB6-8C5	3.9±1.8	19.1±7.8	21.8±4.1	21.6±3.2

a fraction collected from the CCE.
b percentage of positive cells were determined for nonspecific esterase activity and after Diff Quick staining on cell cytospins on slides by counting 400 cells. Percentage of F4/80, MOMA-2, KT3, RA3-6B2, and RB6-8C5 positive cells was determined by flow cytometry.
c NSE: nonspecific esterase, DQ: Diff Quick, F4/80 and MOMA-2: macrophage specific monoclonal antibodies, AF: autofluorescence, KT3: T-cell, RA3-6B2: B-cell and RB6-8C5 granulocyte specific monoclonal antibodies.
The data respresent the mean ± 1 SD of at least six experiments.

previous page

Figure 1. Kupffer cells isolated by counterflow centrifugation elutriation were stained on slides with Diff Quick (A) or non-specific esterase (B). Typical characteristics of the KC, membrane ruffling and large nucleus (N) are shown in the Diff Quick staining (original magnification 1000X). Kupffer cells are stained brown in the non-specific esterase reaction. A sample was taken from Fraction 2 to enhance the differences in non-specific esterase staining (original magnification 400X).

Figure 2. Splenic macrophages isolated by counterflow centrifugation elutriation were stained on slides with (left panel) Diff Quick (2-5, numbers correspond with isolated fractions) or (right panel) non-specific esterase staining of Fraction 5 only (original magnification 200X). Macrophages are characterised by brownish staining in the cytoplasm (arrows), whereas granulocytes (PMN) do not stain with the non-specific esterase reaction (original magnification 400X).

Figure 8. Phagocytosis of *Candida albicans* by Kupffer cells in Fraction 3, 4 and 5. Kupffer cells in a monolayer were exposed to 20 microorganisms per macrophage for 10 min. Fixed monolayers were stained with Diff Quick and 400 cells were examined per monolayer. Phagocytosed microorganisms (arrow head) and nucleus of Kupffer cells (arrow) are visible (original magnification 200X).

The spleen is mostly composed of T- and B-cells. We therefore examined the percentage T-cells (KT3) and B-cells (RA3-6B2) in the different fractions. Neither cell type was removed effectively from the cell populations by CCE (Table 2). The number of T cells in the various fractions did not differ significantly, whereas the percentage of B cells decreased from 63.1% before CCE to 44.3% in Fraction 3. Granulocytes (RB6-8C5) also exhibit phagocytic activity and produce oxygen intermediates. Therefore, the percentage of these cells in the fractions was evaluated. It appeared that the percentage of granulocytes in the spleen cell fractions increased after CCE compared to the complete cell suspension (4.6% before and $\pm$ 20% granulocytes after CCE).

The percentages of cells positive for NSE and positive in the Diff-Quick staining in both the KC and splenic macrophage isolations appeared to correlate closely with the percentages of cells exhibiting AF activity. As can be seen in Table 1 the percentage of AF^+ cells increased from 51.9 to 90.5% between KC fractions 2 and 5. NSE positive cells increased from 41.3 to >95% (correlation coefficient, r^2 0.998). A similar correlation was noted with splenic macrophages with an increase from 0.9 to 14.3% for AF and <1 to 18.3% for NSE (r^2 0.989) . Figs. 3 and 4 illustrate how the percentage of AF cells (macrophages) can be determined by flow cytometry for both liver and spleen.

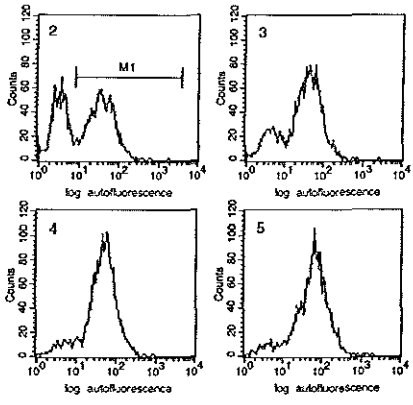

Figure 3. Analysis of Kupffer cell fractions 2-5, collected by counterflow centrifugation elutriation, by flow cytometry using autofluorescence to positively identify macrophages. Cells in the area M1 are AF^+. The graph-numbers correspond to the fraction numbers collected. The graphs are representative of at least six experiments.

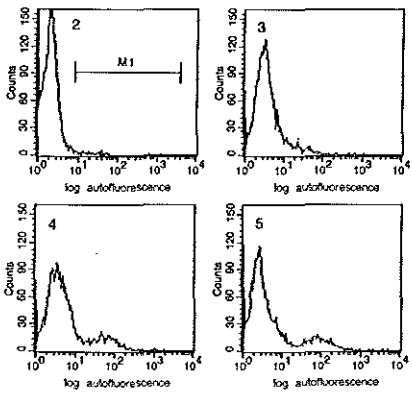

Figure 4. Analysis of spleen macrophage Fractions 2-5, collected by counterflow centrifugation elutriation, by flow cytometry using autofluorescence to positively identify macrophages. Cells in the area M1 are AF^+. The graph-numbers correspond to the fraction numbers collected. The graphs are representative of at least six experiments.

In our hands more macrophages could be isolated from liver and spleen after the addition of DNase and we did not find a deleterious effect of DNase on cell viability in contrast to others (Janousek et al., 1993). In the absence of DNase the isolation procedure resulted in cell clumping, a low yield, and especially for the splenic macrophages in very irreproducible results (Sanderson and Bird, 1977).

Cells were examined for oxygen metabolism in suspension after stimulation with PMA. Figure 5 shows that KC fractions exhibited different levels of oxygen metabolism. Fraction 2 had little metabolic activity (53 000 peak CPM), whereas Fraction 3 was most

active (470 000 peak CPM). Oxygen metabolism decreased again in Fractions 4 and 5 (respectively 290 000 and 190 000 peak CPM). This activity appeared to be superoxide dependent: in all fractions chemiluminescence was decreased by around 70% following the addition of superoxide dismutase (data not shown).

Oxygen metabolism in the spleen cell fractions was much higher compared to the KC cells (Fig. 5). Fraction 3 showed strong activity (1 800 000 peak CPM), which was decreased in Fraction 4 (1 130 000 peak CPM). Fractions 2 and 5 were characterized by low oxygen metabolism compared to the other fractions (110 000 and 290 000 peak CPM respectively). Because of the impurity of the spleen cell fractions and the low yield of splenic macrophages further functional tests were performed only with KC.

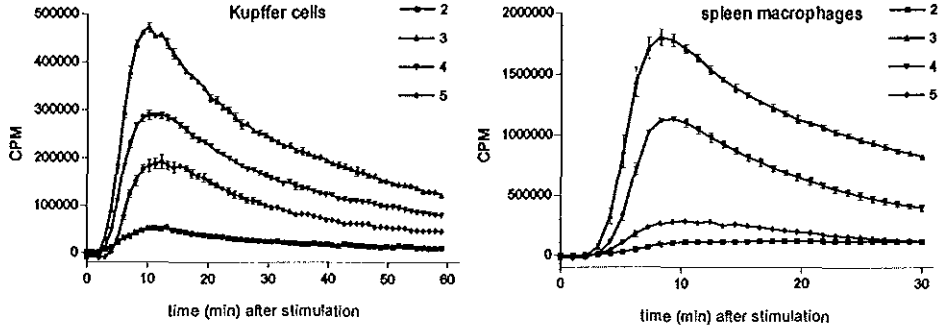

Figure 5. Chemiluminescence of Kupffer cell Fractions 2-5 and spleen macrophage Fractions 2-5 in suspension, collected by counterflow centrifugation elutriation, with PMA used as trigger. Chemiluminescence of unstimulated cells was below the treshold of detection. The data respresent the mean ± 1 SD of three experiments.

The production of significant amounts of NO could only be measured in the culture supernatants of KC Fraction 5 after stimulation with heat killed *Klebsiella pneumoniae*. KC in Fraction 5 produced around 15 μmol NO per 10^6 cells, whereas there was no detectable NO production in supernatants of Fraction 3 (Fig. 6).

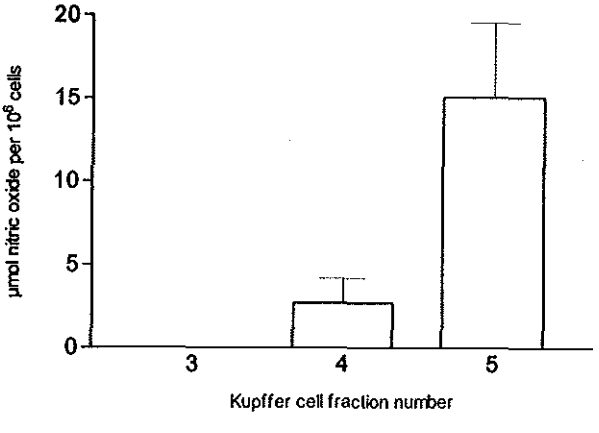

Figure 6. Production of nitric oxide by Kupffer cell Fractions 3, 4 and 5 4 h after stimulation with heat killed *Klebsiella pneumoniae*. Production of NO by unstimulated cells was below the detection level. The data respresents the mean +1 SD of three experiments.

KC purified by CCE and cultured for 6 days were assayed for TNF-α production. Fraction 5 appeared to produce high levels of TNF-α (150 U per 10^6 cells), whereas Fractions 3 and 4 produced small but significant amounts (around 8 U TNF-α per 10^6 cells), after triggering with heat killed *Klebsiella pneumoniae* (Fig. 7).

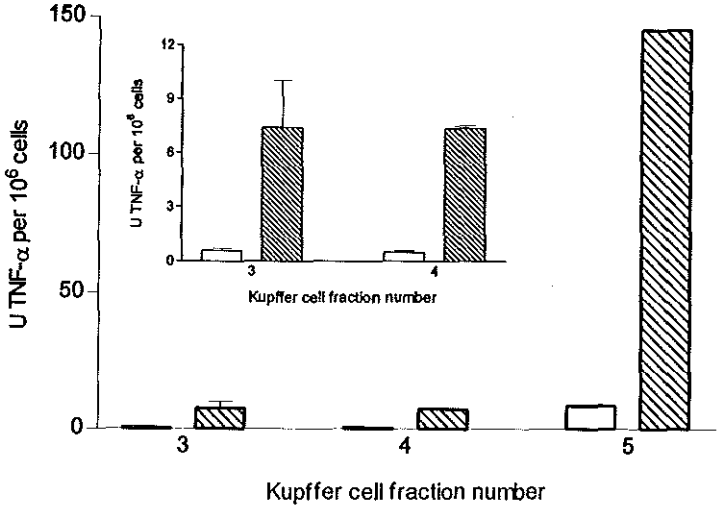

Figure 7. Production of TNF-α by Kupffer cell Fractions 3, 4 and 5 12h after stimulation with heat killed *Klebsiella pneumoniae* (hatched bars), or with medium alone (open bars). The data respresent the mean + 1 SD of three experiments.

All KC fractions exhibited strong phagocytic activity when exposed to *Candida albicans*. After 10 min. of exposure almost 100% of the cells contained microorganisms. KC appeared to phagocytose around 20 microorganisms per macrophage when exposed for 10 min to 50 microorganisms per macrophage (Fig. 8). We observed a TNF-α and RNI production of approximately 60% after two weeks of culture, and all cells still phagocytosed *C. albicans* at that time point (data not shown).

DISCUSSION

In most publications related to the isolation of resident macrophages from liver, KC are considered to be a single population (Heuff et al., 1993; Heuff et al., 1994; Janousek et al., 1994; Knook and Sleyster, 1977; Ogle et al., 1994; Sanders and Soll, 1989). Only few groups have described the isolation of different fractions of macrophages from the liver of the rat (Daemen et al., 1989, Daemen et al.,1991, Hardonk et al., 1989, Itoh et al., 1992, Hoedemakers et al., 1993) and from spleen (Thompson et al., 1983). Cell populations of rat liver macrophages have been functionally examined with emphasis on tumor cytotoxicity and response to immunomodulator treatment (Daemen et al., 1989, Daemen et al., 1991, Hardonk et al., 1989, Hoedemakers et al., 1993) The findings of others in rats and our own (unpublished) observations in vivo in mice, led us to the isolation of subgroups of macrophages from liver and spleen in order to study the heterogeneity of mouse macrophages. In the present study a method is described in which

murine KC and splenic macrophages are isolated and fractionated into three groups of macrophages depending on their size by CCE. It appeared that these cells not only differed in their size but were also characterized by a completely different range of activities.

Three fractions of KC were isolated with purities ranging from 81 to >95% as estimated with NSE and this was confirmed by autofluorescence. However, lower percentages were found when specific mAb were used (F4/80 50.2-56.1% and MOMA-2 61.0-68.2%). This discrepancy was most probably due to (1) the low expression of the surface markers of the macrophages and (2) to the specificity of the mAb resulting in detection of only a subgroup of the macrophages. Furthermore the phagocytosis of *C. albicans* suggested that the fractions contained large numbers of macrophages. The overall yield was around $10X10^7$ cell per isolation, which means that around $1X10^8$ KC were isolated per gram mouse liver. Fraction 3 contained small KC, Fraction 5 contained large, and Fraction 4 contained intermediate sized KC. The isolated KC in the different fractions exhibited different metabolic activities. The production of ROI by KC could not be explained by the presence of granulocytes, because such cells were not detected using our screening methods. However, these findings contrast with those of Lepay et al. (1985), who found KC to be deficient in production of ROI. It is possible that this group selectively isolated large KC which exhibit only very low ROI production.

Although Fraction 3 cells showed strong oxygen metabolism, they produced only very small amounts of TNF-α or nitric oxide after stimulation. Fraction 4 was comparable to Fraction 3, but Fraction 5, which was composed of large KC, showed a significant production of TNF-α and nitric oxide. These results are comparable with those of Decker et al. (1989) and Ogle et al. (1994), suggesting that their KC population contained predominantly large KC. However, Shimauchi et al. (1993) reported that KC produced much lower amounts of TNF–α, which might be explained by a KC population composed of predominantly small or intermediate cells. We found that an improved separation could be obtained between the KC fractions when the rotor speed was set to 3200 rpm, compared to the generally used setting of 2400 rpm (unpublished observations). We also used this setting for splenic macrophage isolation.

The isolation of murine splenic macrophages proved far more difficult. Various fractions were collected by CCE and increasing percentages of macrophages from 4% up to 11.4%, 21.8%, and 21.9% respectively were obtained in the last three fractions as detected with mAb F4/80. Flow cytometry using the MOMA-2 antibody yielded comparable percentages of 6.4, 19.6, and 19.4 respectively. These finding are in agreement with Buckley et al. (1984), who isolated one fraction from human spleen with 28% mononuclear phagocytes, but they differ from those of Thompson et al. (1983). In the latter study various fractions were isolated but the percentage of macrophages ranged from 3 to 5.2%. In the spleen mAb F4/80 predominantly recognizes red pulp macrophages, whereas MOMA-2 binds to (mature) white pulp macrophages, as well as to red pulp macrophages (review by Leenen et al., 1994).

Production of ROI by splenic macrophages in the different fractions was similar to that observed with the KC fractions. However, the interpretation of these results are hampered by the presence of granulocytes in the fractions, which exhibit strong oxygen metabolism. It appeared that the percentage of granulocytes in the last three fractions were practically the same, which suggests that the percentage of granulocytes does not account for the differences in ROI production. It is unclear whether the differences in metabolic activity in the three fractions were related to differences in the macrophages, or whether size separation of granulocytes also occurs resulting in granulocytes with different oxygen metabolism.

REFERENCES

Austyn, J.M. and Gordon, S. (1980) F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur. J. Immunol. 11, 805.

Buckley, P.J., Beelen, R.H.J., Burns, J., Beard, C.M., Dickson, S.A. and Walker W.S. (1984) Isolation of human splenic macrophages and lymphocytes by countercurrent centrifugal elutriation. J. Immunol. Methods 66, 201.

Coffman, R.L. (1982) Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B development. Immunol. Rev. 69, 5.

Daemen, T., Veninga, A., Regts, J., and Scherphof, G.L. (1991) Maintenance of tumoricidal activity and susceptibility to reactivation of subpopulations of rat liver macrophages. J. Immunother. 10, 200.

Daemen, T., Veninga, A., Roerdink, F.H., and Scherphof, G.L. (1989) Endocytic and tumoricidal heterogeneity of rat liver macrophage populations. Sel. Cancer Ther. 5,157.

Decker, T., Lohman-Matthes, M-L., Karck, U., Peters, T., and Decker, K. (1989) Cooperative study of cytotoxicity, tumor necrosis factor, and prostaglandin release after stimulation of rat Kupffer cells, murine Kupffer cells, and murine inflammatory liver macrophages. J. Leukocyte Biol. 45, 139.

Hardonk, M.J., van Goor, H., Scherphof, G.L., and Daemen, T. (1989) Functional heterogeneities between large and small Kupffer cells. In: E. Wisse, D.L. Knook, and K. Decker (Eds.), Cells of the Hepatic Sinusoid., Vol 2; Proc. Fourth Int. Symp. Cells Hepatic Sinusoid, Titisee 1989. The Kupffer Cell Foundation, Leiden, The Netherlands, p434.

Espevik, T. and Nissen-Meyer J. (1986) A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J. Immunol. Methods 95, 99.

Heuff, G., Meyer, S. and Beelen, R.H.J. (1994) Isolation of rat and human Kupffer cells by a modified enzymatic assay. J. Immunol. Methods 174, 61.

Heuff, G., Steenbergen, J.J.E., Vandeloosdrecht, A.A., Sirovich, I., Dijkstra, C.D., Meyer, S. and Beelen, R.H.J. (1993) Isolation of cytotoxic Kupffer cells by a modified enzymatic assay - A methodological study. J. Immunol. Methods 159, 115.

Hoedemakers, R.M., Vossebeld, P.J., Daemen, T. and Scherphof, G.L. (1993) Functional characteristics of the rat liver macrophage population after single intravenous injection of liposome-encapsulated muramyl peptides. J. Immunother. 13(4), 252.

Humphrey, J.H. and Grennan, D. (1981) Different macrophage populations distinghised by means of fluorescence polysaccharides. Recognition and properties of marginal-zone macrophages. Eur. J. Immunol. 11, 221.

Itoh, Y., Okanoue, T., Morimoto, M., Nagao, Y., Mori, T., Hori, N., Kagawa, K., and Kashima, K. (1992) Functional heterogeneity of rat liver macrophages: Interleukin-1 secretion and Ia antigen expression in contrast with phagocytic activity. Liver 12, 26.

Janousek, J., Strmen, E. and Gervais F. (1993) Purification of murine Kupffer cells by centrifugal elutriation. J. Immunol. Methods 164, 117.

Knook, D.L. and Sleyster, E.Ch. (1977) Preparation and characterization of Kupffer cells from rat and mouse liver. In: E. Wisse, and D.L. Knook (Eds.), Kupffer Cells and Other Liver Sinusoidal Cells; Proc. Int. Kupffer Cell Symp., Noordwijkerhout, 1977. Elsevier, New York, p. 273.

Kraal, G., Rep, M. and Janse, M. (1987) Macrophages in T and B cell compartments and other tissue macrophages recognized by monoclonal antibody MOMA-2. An immunohistochemical study. Scand J. Immunol. 26, 661.

Ledbetter, J.A. and Herzenberg, L.A. (1979) Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47, 63.

Leenen, P.J.M., de Bruijn, M.F.T.R., Voerman, J.S.A., Campbell, P.A., and van Ewijk, W. (1994) Markers of mouse macrophage development detected by monoclonal antibodies.
J. Immunol. Methods 174, 5.

Lepay, D.A., Nathan, C.F., Steinman, R.M., Murray, H.W., and Cohn, Z.A. (1985) Murine Kupffer cells. Mononuclear phagocytes deficient in the generation of reactive oxygen intermediates. J. Exp. Med. 161, 1079.

Ogle, C.K., Wu, J.Z., Ma, X.L., Szczur, K., Alexander, J.W. and Ogle, J.D. (1994) Heterogeneity of Kupffer cells and splenic, alveolar, and peritoneal macrophages for the production of TNF, IL-1, and IL-6. Inflammation 18, 523.

Redmond, H.P., Shou, J., Gallagher, H.J., Kelly, C.J. and Daly, J.M. (1993) Macrophage-dependent candidacidal mechanisms in the murine system - Comparison of murine Kupffer cell and peritoneal macrophage candidacidal mechanisms. J. Immunol. 150, 3427.

Sanders, J.M. and Soll, A.H. (1989) Cell separation by elutriation: Major and minor cell types from complex tissues. Methods Enzymol. 171, 482.

Sanderson, R.J. and Bird, K.E. (1977) Cell separations by counterflow centrifugation. Methods Cell Biol.

15, 1.

Shimauchi, Y., Tanaka, M., Yoshitake, M., Shimada, M., Sato,K., Kuromatsu, R, Tanaka, S., Kumashiro, R., Sakisaka, S. and Tanikawa, K. (1993) Functional differences between rat Kupffer cells and splenic macrophages. In: K. Decker, D.L. Knook and E. Wisse (Eds.), Cells of the Hepatic Sinusoid, Vol 4; Proc. Sixth Int. Symp. Cells Hepatic Sinusoid, Antwerp, 1992. The Kupffer Cell Foundation, Leiden, The Netherlands, p 198.

Stroben, W. (1994) Wright-Giemsa and Nonspecific Esterase Staining of Cells. In: J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach and W. Strober (Eds.), Current Protocols in Immunology, Vol 2. Greene Publishing Associates, Inc. And John Wiley & Sons, Inc. p A3.5.

Stuehr, D.J. and Nathan, C.F. (1989) Nitric oxide a macrophage product responsible for cytostasis and respiratory inhibition of tumor target cells. J. Exp. Med. 169, 1543.

Thompson, C.B., Ryan, J.J., Sieckmann, G., Finkelman, F.D., Mond, J.J. and Scher, I. (1983) A method for size separation of murine spleen cells using counterflow centrifugation. J. Immunol. Methods 63, 299.

Thorell, B. (1981) Flow cytrometric analysis of cellular endogenous autofluorescence simultanously with emission from exogenous fluorochromes, light scatter, and absorption. Cytometry 2, 39.

Tomonari, K. (1988) A rat antibody against a structure functionally related to the mouse T-cell receptor/T3 complex. Immunogenetics 28, 455.

CHAPTER EIGHT

Differential Nitric Oxide and TNF–α Production of Murine Kupffer Cell Subfractions Upon Priming With IFN–γ And TNF–α.

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ABSTRACT

Heterogeneity of murine Kupffer cells (separated on size in three fractions) after priming with macrophage activating cytokines in response to bacteria is demonstrated in the present study. Striking heterogeneity of naive (unprimed) Kupffer cells (KC) depending on cell size has been shown before. However, a shift in responses was observed in KC fractions separated on cell size by counter current elutriation upon priming with tumor necrosis factor–α (TNF–α) or interferon–γ (IFN–γ). Whereas unprimed large KC are most active in production of TNF–α and nitric oxide (NO), after priming of KC with TNF–α predominantly small and intermediate sized KC produce TNF–α in response to bacteria. Priming with IFN–γ enhanced NO prodcution in all KC. Strong synergy was observed when KC subfractions were exposused to combination of TNF–α and IFN–γ with respect to production of NO. However, IFN–γ seemed to exert an inhibitory effect on TNF–α priming. The dramatically increased TNF–α production of macrophages primed with TNF–α was strongly diminished by the addition of IFN–γ.

The present data demonstrate clear heterogeneity among murine KC with respect to immunologic response to stimuli. These results demonstrate that KC have different functions in immunologic reactions which seem to be related to size.

INTRODUCTION

The resident macrophages of liver (Kupffer cells) have been extensively studied with respect to their functions in vivo and in vitro. Although Kupffer cells (KC) are described mostly as a single population several studies demonstrate heterogeneity among these cells (1, 2, 3, 4). We recently investigated heterogeneity of murine KC with respect to oxygen metabolism, TNF–α production and NO release after size fractionation by counter current elutriation (CCE) (5). Others found that rat KC fractionated on size exhibit heterogeneity with respect to cytotoxicity, IL-1 production, antigen expression and phagocytosis (1, 2, 3, 4). In our previous study we found that naive large KC produced significantly more TNF–α and NO than intermediate KC or small KC (5).

Due to their relative large number and localization KC occupy an important role in blood clearance and regulation of immune reactions. In vitro studies examining crude unseparated macrophage populations, demonstrated that macrophage activity can be increased by exposure to TNF–α and IFN–γ (6). Also in vivo it is likely that KC become pre-activated by cytokines and other signal peptides and therefore demonstrate augmented activity when subsequently exposed to secondary stimuli. TNF–α and IFN–γ play an important role in this process. The aim of our study was to examine heterogeneity of KC during an immunologic reaction. Thereto KC were separated on size, primed with TNF–α and IFN–γ and exposed to bacteria in vitro, and their response studied.

MATERIALS AND METHODS

Animals. Specified pathogen free, 13 to 15 weeks old male C57Bl/Ka mice were used (ITRI-TNO, Rijswijk, The Netherlands). Animal care was performed according to institutional guidelines.

Cytokines. Recombinant murine IFN–γ was derived from supernatant fluid of Mick cells, a CHO cell line that carries and expresses an amplified murine IFN–γ cDNA (7). Murine IFN–γ was purified by affinity chromatography on an anti-rat IFN–γ mAb (DB-1) to a specific activity of $6X10^6$ U/mg, and used at a concentration of 500 U/ml. Recombinant murine TNF–α was kindly provided by Dr G.R. Adolf (Bender Wien, Vienna, Austria), and used at a concentration of 0.5 μg/ml.

Bacteria. Klebsiella pneumoniae, capsular serotype 2 (ATCC 43816), was used. Bacteria were grown for 16 h at 37°C in Todd-Hewitt broth (Oxoid Ltd, Basingstoke, UK), heat killed (15 min. 121°C) and washed three times in PBS.

Kupffer cell isolation. Isolation of Kupffer cell subfractions was performed as previously described (5). Briefly, after in situ perfusion of liver with collagenase A solution (Boehringer Mannheim, Mannheim, FRG), liver was excised from mice. Livers of 8 mice were incubated in collagenase A solution at 35°C for 10 min, and then gently minced and filtered through a nylon gauze (212 μm). Liver cell suspensions were fractionated on a Nycodenz gradient (Nycomed Pharma As, Oslo, Norway) to remove parenchymal cells. Non-parenchymal cells were further separated into various fractions by CCE using the autoclavable Beckman JE-5.0 elutriator rotor supplied with a Sanderson chamber (Beckman Instruments Inc, Palo Alto, CA, USA).

Culture of isolated Kupffer cells. Isolated Kupffer cells from Fractions 3, 4, and 5 were brought to a density of $2.0X10^6$ cells per ml in DMEM high glucose culture medium (GIBCO, Life Technologies Inc. Gaithersburg, MD), supplemented with 15% FCS (GIBCO). Cells were cultured in 96 well plates at a density of $1.5X10^5$ cells per well. Kupffer cells were cultured in triplicate, exposed to IFN–γ, TNF–α or to both for 24 h, after which the cells were washed three times. Cells were taken up in DMEM supplemented with 10% FCS (TNF–α determination) or HBSS with 0.01% gelatin (NO quantification).

Assessment of TNF–α production by Kupffer cells. Briefly, $1.5X10^5$ macrophages in 150 μl DMEM supplemented with 10% FCS, were exposed to heat killed *K. pneumoniae* in a ratio of 15 bacteria per macrophage for 4 h, whereafter 50 μl supernatant samples were taken. TNF–α levels were determined in culture medium using the WEHI 164 bioassay (8).

Quantification of production of nitrogen (RNI) intermediates. Nitrite production was measured using Griess reagents as reported by Stuehr et al. (9). Briefly, $1.5X10^5$ macrophages in 150 μl HBSS with 0.01% gelatin, were exposed to heat killed *K. pneumoniae* in a ratio of 15 bacteria per macrophage for 2 h, whereafter 150 μl of Griess reagents was added. Absorption at 570 nm was determined and compared with a standard curve prepared at the same time using known concentrations of sodium nitrite. Production of NO below 1.5 μmol per 10^6 cells were considered unreliable due to the detection limit of the assay.

Statistical analysis. Groups were compared using Kruskal-Wallis analysis. *P*-values below 0.05 were considered significant.

RESULTS

Production of TNF–α by murine Kupffer cell subfractions. Figure 1 shows dramatic heterogeneity of unprimed KC in TNF–α production when stimulated in vitro with heat killed *K. pneumoniae*. Whereas KC in Fraction 3 and 4 only produced small quantities of TNF–α, KC in Fraction 5 produced up to 135 U TNF–α per 10^6 cells upon stimulation.

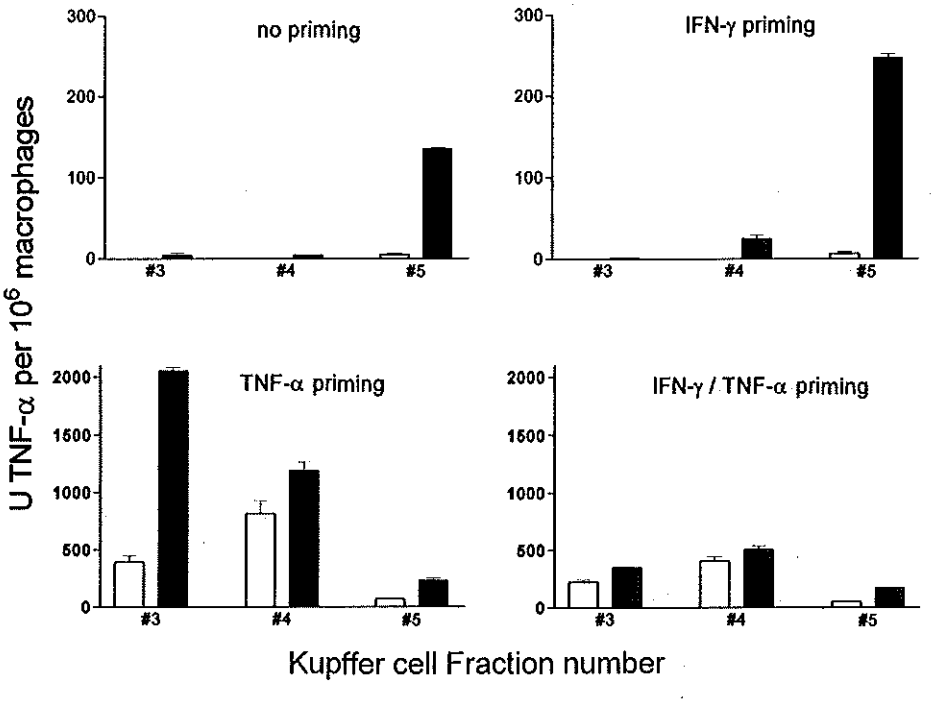

Figure 1. Production of TNF–α by murine Kupffer cells in vitro after priming with TNF–α and/or IFN–γ for 24 h and subsequently stimulated with heat killed *Klebsiella pneumoniae* (■) or not stimulated (□). Kupffer cells were isolated in three fractions (small: 3, intermediate: 4, and large: 5) from eight pooled livers. Bars represent the average of three determinations + SD.

After priming of KC for 24 h with 500 U IFN–γ per $1.5X10^5$ cells KC were stimulated with heat killed *K. pneumoniae*. Although KC in Fraction 3 still failed to produce measurable amounts of TNF–α, pronounced 6-fold increase to 25 U TNF–α per 10^6 cells was seen in Fraction 4 compared with unprimed KC ($p < 0.05$). Priming of KC in Fraction 5 with IFN–γ resulted in 1.8-fold increase in production of TNF–α compared with unprimed macrophages ($p < 0.05$). Primed KC not stimulated with *K. pneumoniae* did not produce significant TNF–α levels.

Priming of KC for 24 h with TNF–α resulted in high levels of TNF–α in supernatants of all KC subfractions, both unstimulated and stimulated with *K. pneumoniae*. Exposure of KC to TNF–α resulted especially in KC Fraction 4 in high TNF–α production. However, priming of cells followed by stimulation with *K. pneumoniae* resulted in dramatic induction of TNF–α production in especially Fraction 3, whereas KC in Fraction 5 only produced relatively small amounts of TNF–α.

TNF–α production was also measured in the KC subfractions exposed for 24 h to both IFN–γ and TNF–α. Priming of the KC with the cytokines resulted in reduced TNF–α

production in all subfractions compared to the cells primed with TNF–α alone. Stimulation of these KC with heat killed bacteria had only little but significant enhancing effect on TNF–αproduction compared with unstimulated cells. Taken together these results indicate that especially TNF–α has potent enhancing effect on in particular small and intermediate sized KC. Whereas IFN–γ especially activates large KC and moreover, seems to inhibit the activity of TNF–α on KC.

Production of nitric oxide by murine Kupffer cell subfractions. Figure 2 shows production of significant amounts of NO by unprimed KC in Fraction 5 after stimulation with heat killed *K. pneumoniae*. Priming however with IFN–γ augmented NO production by all KC subfractions significantly upon stimulation with *K. pneumoniae*. NO production was still highest in Fraction 5.

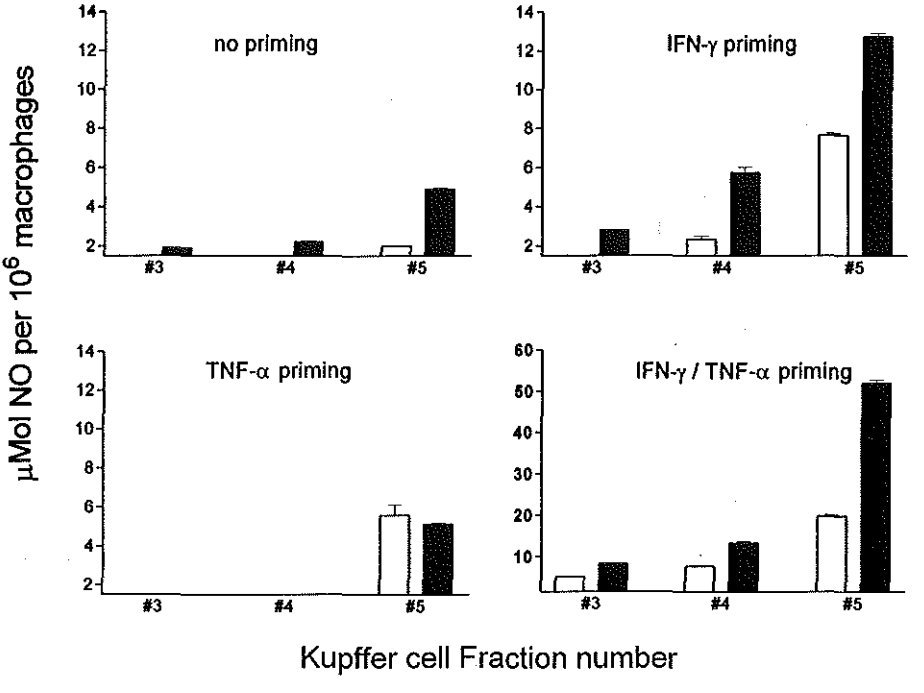

Figure 2. Production of nitric oxide (NO) by murine Kupffer cells in vitro after priming with TNF–α and/or IFN–γ for 24 h and subsequently stimulated with heat killed *Klebsiella pneumoniae* (■) or not stimulated (□). Kupffer cells were isolated in three fractions (small: 3, intermediate: 4, and large: 5) from eight pooled livers. NO production is measured as the amount of nitrite (NO_2^-) present in the culture medium. Bars represent the average of three determinations + SD.

Priming of KC subfractions for 24 h with TNF–α had less enhancing effect, but significantly induced NO production already in unstimulated cells in Fraction 5. Strikingly, stimulation of these cells with *K. pneumoniae* after TNF–α priming did not significantly change NO production in Fraction 5, or other fractions.

Priming of KC with both IFN–γ and TNF–α resulted in a strong up regulation of NO production in all subfractions. Most dramatic enhancement of NO production by priming with IFN–γ and TNF–α was observed in Fraction 5. In unstimulated cells 20 μmol NO was produced per 10^6 cells, whereas *K. pneumoniae* stimulated cells produced up to 52 μmol of NO per 10^6 cell. A 2.6-fold increase compared with IFN–γ/TNF–α primed unstimulated cells ($p < 0.05$), but an increase of 10.4 fold compared to unprimed *K. pneumoniae* stimulated cells in Fraction 5 as represented in figure 2a.

Taken together these results demonstrate that IFN–γ especially up regulated NO production in response to bacteria in intermediate and large sized KC, whereas priming with TNF–α had only effect on large KC. However, strong synergy between IFN–γ and TNF–α in activation for NO production was observed in all KC subfractions.

DISCUSSION

In this paper we examined the heterogeneity of Kupffer cells upon priming with important macrophage activating cytokines: TNF–α and IFN–γ. We previously demonstrated that naive KC are very heterogeneous with respect to production of TNF–α and NO (5). Especially large KC produced TNF–α and NO after stimulation with heat killed *K. pneumoniae*. In the present paper we demonstrate that priming of KC subfractions with IFN–γ and TNF–α had strong augmenting effect on the activity of isolated KC. Exposure of KC to IFN–γ led to increased production of TNF–α and NO especially in intermediate and large sized KC (Fractions 4 and 5), whereas priming with TNF–α had a dramatic enhancing effect on TNF–α production by small and intermediate sized KC (Fractions 3 and 4). However, priming with TNF–α had only slight effect on production of NO in all subfractions. Combination of TNF–α and IFN–γ resulted in increased activity in all subfractions, TNF–α production being highest in Fraction 4, and NO production highest in Fraction 5. Strikingly, TNF–α production by KC exposure to TNF–α alone was much higher than by KC primed with both TNF–α and IFN–γ. On the other hand, priming with TNF–α and IFN–γ had a synergistic effect on production of NO by all KC subfractions when stimulated with heat killed bacteria.

TNF–α is known to have strong activating effect on macrophages in an autocrine and paracrine fashion. Exposure of macrophages to IFN–γ or TNF–α results in increased production of oxygen and nitrogen intermediates, increased killing of intracellular microorganisms and increased cytotoxic activity, but also strong synergy between cytokines has been observed (6). In vivo macrophages encountering a foreign substance will respond with phagocytosis of the substance and increased production of monokines and metabolic activity. Through production of TNF–α other macrophages are directly or indirectly activated (10). T-cells, activated by macrophage derived monokines (i.e IL-1), produce IFN–γ which in turn has activating effects on macrophages. We demonstrated that both TNF–α and IFN–γ prime macrophages respond to stimulation with heat killed bacteria with an increased TNF–α and NO production in a heterogeneous way. It is striking that predominantly large KC demonstrated NO and TNF–α production when unprimed, whereas small and intermediate KC were superior in TNF–α production after

exposure to the cytokines.

It is tempting to speculate that when KC are confronted with microorganisms in vivo, large KC are the first to respond with phagocytosis of the organisms, followed by production of TNF–α, IL-1 and other signal peptides. These in turn primes small and intermediate KC to enhanced response when exposed to microorganisms or their products. Others showed that especially large KC reside in the periportal area, whereas small KC predominantly localize in the perivenous area (1, 11). They demonstrated that large KC are predominantly involved in phagocytosis of foreign substances in blood, and suggested that KC functional heterogeneity is related to their position in the liver acinus, which is supported by our findings.

The heterogeneity observed in the present study in murine KC correlates with the observed heterogeneity of rat KC. Others found that especially large KC are responsible for phagocytosis, whereas small and intermediate KC demonstrated more IL-1 production and Ia expression (4). However, they failed to demonstrate heterogeneity in TNF–α production. These findings seem in contradiction with ours, but are probably due to isolation procedure or stimulation level of macrophages. KC isolated from rat treated with macrophage activating agents (muramyl peptides) showed heterogeneity with respect to tumor cytotoxicity in vitro (3, 12, 13, 14). Small KC seemed superior in killing of tumor cells, which is in agreement with our findings that especially small KC produce TNF–α when primed.

Although highly speculative, large KC seem to be responsible for the onset of host defense reaction, whereas smaller sized KC are effector cells. It is not clear however, whether small KC when activated develop into large KC (being a matter of maturation) or whether these cells make up different types of macrophages. Our findings however do make clear that KC heterogeneity has to be taken into account when host defense activating is studied.

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REFERENCES

1 Bouwens L, Baekeland h, de Zanger R, Wisse E. Quantitation, Tissue distribution and proliferation kinetics of Kupffer cells in normal rat liver. Hepatology 1986;6:718-722.

2 Daemen T, Veninga A, Roerdink FH, Scherphof GL. Endocytic and Tumoricidal heterogeneity of rat liver macrophage populations. Selective Cancer Therapeutics 1989;5:157-167.

3 Hoedemakers RMJ, Morselt HWM, Scherphof GL, Daemen T. Secretion pattern of the rat liver macrophage population following activation with liposomal muramyl dipeptide in vivo and in vitro. Journal of Immunotherapy 1994;15:265-272.

4 Itoh Y, Okanoue T, Morimoto M, Nagao MY, Mori T, Hori TN, Kagawa K, Kashima K. Functional heterogeneity of rat liver macrophages: interleukin-1 secretion and Ia antigen expression in contrast with phagocytic activity. Liver 1992;12:26-33.

5 Hagen TLM, Van Vianen W, Bakker-Woudenberg IAJM. Isolation and characterization of murine Kupffer cells and splenic macrophages. Journal of Immunological Methods 1996;193:81-91.

6 Sheehan KCF, Schreiber RD. The synergy and antagonism of interferon–γ and TNF. In: Beutler B, Ed. Tumor Necrosis Factor, the molecules and their emerging role in medicine. Raven Press, N.Y. 1992:145-178

7 Dijkmans R, Heremans H, Billiau A. Heterogeneity of chinese hamster ovaria cell-produced recombinant murine inerferon–γ. Journal of Biological Chemistry 1987;262:2528.

8 Espevik T, Nissen-Meyer J. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic

factor/tumor necrosis factor from human monocytes. Journal of Immunological Methods 1986;95:99-105.

9 Stuehr DJ, Nathan CF. Nitric oxide a macrophage product responsible for cytostasis and respiratory inhibition of tumor target cells. Journal of Experimental Medicine 1989;169: 1543-1555.

10 Cockfield SM, Ramassar V, Halloran PF. Regulation of IFN-γ and Tumor Necrosis Factor-α expression in vivo. Journal of Immunology 1993;150:342-352.

11 Sleyster E Ch, Knook DL. Relation between localization and function of rat Kupffer cells. Laboratory Investigation 1982;47:484-490.

12 Daemen T, Veninga A, Regts J, Scherphof GL. Maintenance of tumoricidal activity and susceptibility to reactivation of subpopulations of rat liver macrophages. Journal of Immunotherapy 1991;10:200-206.

13 Daemen T. Activation of Kupffer cell tumoricidal activity by immunomodulators encapsulated in liposomes. Research in Immunology 1992;143:211-214.

14 Hoedemakers RMJ, Vossebeld PJM, Daemen T, Scherphof GL. Functional characteristics of the rat liver macrophage population after a single intravenous injection of liposome-encapsulated muramyl peptides. Journal of Immunotherapy 1993;13: 252-260.

CHAPTER NINE

GENERAL DISCUSSION

Immunomodulation With MDP Analogues and IFN-γ in Experimental Gram-negative Septicemia: Emphasis on the use of liposomes as drug carriers.

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CONTENTS

1. INTRODUCTION

Severe infections represent a continuing threat to immunocompromised patients. The availability of a broad spectrum of antibiotics and the large number of studies on optimal dosing and administration protocols have not taken this threat away. Gram-negative septicemia, a relatively rare clinical diagnosis a few decades ago, is perhaps the most important infectious disease problem in hospitals today. Even the recent shift in infection type from Gram-negative to Gram-positive, has not diminished the mortality due to Gram-negative infections. Among patients who develop complications of shock and organ failure, mortality can reach 90%. Septicemia thus represents a leading cause of death in the developed countries, and its incidence has increased significantly over the past decade (1).

Factors considered important in development of septicemia include broad-spectrum antibiotic therapy, immunosuppressive treatments (used in cancer, transplantation, autoimmune diseases, and chronic inflammations), use of invasive devices and surgery, burns or other traumata, presence of intestinal anatomic obstruction or ulceration, advanced age or immunologic immaturity in the young, chronic underlying conditions (malignancies, diabetes, AIDS) and other serious chronic diseases (1). In such patients antimicrobial therapy of septicemic infection often fails.

Cure rates from infection is poorest among those patients who have persistent neutropenia. Various strategies are available to improve treatment of immunodeficient patients with a severe infections. One strategy is to intensify antibiotic treatment by applying more drugs at the same time; this is however not always effective. Poor results may at least partly be due to occurrence of antimicrobial resistance among microorganisms causing septicemia. The inability to eliminate all microorganisms from the focus of infection results in emergence of resistant microorganisms, and recurrence of infection. Failure of host defense to support antibiotic treatment allows this to happen. Another possible way to improve therapeutic results might thus be to stimulate host defense, either nonspecific defense (granulocytes and macrophages) or specific humoral and cellular defense.

Activation of nonspecific host defense has the advantages that it can be effective in different types of infection, and does not induce tolerance of the microorganisms to treatment. Cells of the mononuclear phagocyte system (MPS) play a key role in nonspecific host defense. Activation of these cells will result first of all in enhanced killing of intracellular microorganisms infecting the MPS. However, it may be expected that activation of the MPS will enhance resistance to more systemic (extracellular) infections as well. Increased blood clearance and microbial killing capacity of the MPS as well as an enhanced granulocyte blood count may result from treatment with so-called immunomodulators. Activation of nonspecific host defense can be achieved with biologic or synthetic immunomodulators that influence or modify (parts of) the innate resistance in a direct or indirect way, independently of challenge. Many different agents have been tested for their immunomodulatory capacity.

The immunomodulatory agents are of natural origin, for instance extracts of bacterial or herbal origin and cytokines, or synthetic (for instance some of the muramyl peptide derivatives). The introduction of recombinant DNA technology has paved the way for the use of cytokines as immunomodulatory agents. Large quantities of relatively pure

cytokine can be produced under controlled circumstances. This review is focussed in particular on results obtained with muramyldipeptide (MDP) and its derivative muramyl tripeptide phosphatidylethanolamine (MTPPE), and the cytokine interferon-γ (IFN-γ). Although muramyl peptide derivatives are under investigation in phase I/II trials in cancer patients to study their effects on malignancies, no clinical trails have as yet been performed to study their effects on infections. However, macrophage stimulating- and antimicrobial activities of MDP (and derivatives) and IFN-γ to a broad spectrum of microorganisms have already been shown in vitro and in vivo (table 1). Thusfar promising studies with IFN-γ in chronic granulomatous disease (CGD) patients have led to the approval of IFN-γ for prophylaxis against opportunistic infections in these patients (60). Moreover, as will be discussed in more detail, data have been obtained supporting the possible usefulness of IFN-γ for prophylaxis in other groups of patients.

2. THE IMMUNOMODULATORS

MDP, which is part of the peptidoglycan of the cell wall of bacterium *Mycobacterium* BCG, is the minimum adjuvant-active compound in Freund's complete adjuvant. It can be produced synthetically, and many modifications have been made to reduce its toxicity or improve its activity and usefulness. The derivative under investigation by us is MTPPE, a synthetic hydrophobic derivative of MDP, in the liposome-encapsulated form (LE-MTPPE). MTPPE shows, as will be discussed below, improved activity over MDP and can easily be incorporated into liposomes. IFN-γ is a cytokine primarily produced by stimulated Th1 cells, $CD8^+$ T cells and NK cells. It infuences all major macrophage functions including MHC II expression, antigen presentation, FcγR1 receptor expression, uptake and intracellular killing of microorganisms, tumor cell cytotoxicity, and the production of monokines (61-66).

2.1. IN VITRO STUDIES

Both muramyl peptides and IFN-γ were found to have a potent enhancing effect on macrophage ability to kill microorganisms (table 1). Also for several viral infections enhanced killing after stimulation with these immunomodulators was shown (table 1). The agents were found to induce the production of reactive oxygen and nitrogen intermediates (ROI and RNI), and other antimicrobial substances by macrophages, explaining the enhanced antimicrobial activity of the infected cells (Fig. 1). Therefore, the rationale to use these immunomodulators for activation of host defense to intracellular infections in vivo is self-evident. However, immunomodulators can also enhance macrophage activity against extracellular infections. Table 2 shows an overview of studies on immunomodulatory activity of MDP/MTPPE or IFN-γ to Gram-negative bacteria capable to induce septicemia. Increased uptake and killing of *Pseudomonas aeruginosa* by macrophages exposed to IFN-γ in vitro was noticed (135). Treatment of human peripheral blood monocytes with IFN-γ in vitro greatly enhanced both respiratory burst and microbicidal activity elicited by *P. aeruginosa* (14). However, others found no effect on

Table 1 In vitro and in vivo studies on antimicrobial enhancing activity of muramyl dipeptide (MDP), muramyl tripeptide phosphatidyl ethanolamine (MTPPE) and IFN-γ, in the free or liposome-encapsulated (LE) form.

Micro-organism	Immunomodulators and Refs			
FUNGI				
Aspergillus fumigatus			IFN-γ	(2, 3)
Blastomyces dermatitidis			IFN-γ	(4, 5, 6)
Candida albicans	MDP/LE-MDP/MTPPE	(7-10)	IFN-γ	(3, 6, 11-15)
Candida parapsilosis			IFN-γ	(3)
Cryptococcus neoformans			IFN-γ	(3, 6)
Coccidioides immitis			IFN-γ	(3, 6, 16, 17)
Histoplasma capsulatum			IFN-γ	(3, 6)
Paracoccidioides brasiliensis			IFN-γ	(3, 6)
Pneumocystis carinii			IFN-γ	(3, 6)
INTRACELLULAR BACTERIA				
Brucella abortus			IFN-γ	(3)
Chlamydia psittaci			IFN-γ	(3, 6, 18, 19)
Chlamydia trachomatis			IFN-γ	(3,6)
Francissella tularensis			IFN-γ	(3, 6)
Legionella pneumophila			IFN-γ	(3, 6, 20-23)
Listeria monocytogenes	MDP	(24, 25)	IFN-γ/LE-IFN-γ	(3,6,11,26-34)
Mycobacterium avium	MDP	(35)	IFN-γ	(6, 36)
Mycobacterium bovis			IFN-γ	(3, 6, 37)
Mycobacterium microti			IFN-γ	(3, 6, 32)
Mycobacterium tuberculosis			IFN-γ	(3, 6, 38, 39)
Nocardia asteroides			IFN-γ	(3, 6)
Rickettsia prowazekii			IFN-γ	(3, 6)
Rickettsia conori			IFN-γ	(3, 6)
Rickettsia tsutsugamushi			IFN-γ	(3, 6)
Salmonella enteritidis	see table 2			
Salmonella typhimurium	see table 2			
EXTRACELLULAR BACTERIA				
Escherichia coli	see table 2			
Klebsiella pneumoniae	see table 2			
Naegleria fowleri	MDP	(40)		
Pseudomonas aeruginosa	see table 2			
Staphylococcus aureus			IFN-g	(3,41, 42)
Streptococcus pneumoniae	MDP	(15)		
Yersinia enterocolitica			IFN-γ	(3)
VIRUSES				
Friend leukemia virus	MDP/MTPPE/LE-MTPPE	(7)		
herpes simplex virus	MDP/MTPPE/LE-MTPPE	(7, 43)	IFN-γ	(44)
human immunodeficiency virus	MDP	(45)		
influenza A,B	MTPPE/LE-MTPPE	(7, 46-48)		
murine hepatitis virus	MDP	(49)		
parainfluenza	MTPPE	(7)		
Rift Valley fever virus	MTPPE	(7, 50, 51)		
PROTOZOA				
Cryptosporidium parvum			IFN-γ	(3)
Entamoeba histolytica			IFN-γ	(3, 6)
Giardia lamblia			IFN-γ	(3)

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Leishmania donovani	LE-MTPPE	(52)	IFN-γ	(3, 6, 18, 39, 53, 52)
Leishmania major			IFN-γ	(3, 6)
Leishmania mexicana			IFN-γ	(3, 6)
Leishmania enrietii			IFN-γ	(3)
Plasmodium falciparum			IFN-γ	(3, 6)
Plasmodium vivax			IFN-γ	(3, 6)
Plasmodium berghei			IFN-γ	(3, 6)
Plasmodium chabaudi			IFN-γ	(3, 6)
Plasmodium cynomolgi			IFN-γ	(3, 6)
Trypanozoma cruzi	MDP	(40, 54)	IFN-γ	(3, 5, 6, 55, 56)
Toxoplasma gondii	MDP	(40)	(LE)-IFN-γ	(3, 6, 57, 58)
HELMINTHS				
Schistosoma mansoni	MTPPE	(59)	IFN-γ	(3, 6)
Ehrlichia risticii			IFN-γ	(3)

Effect Immunomodulators on Macrophage

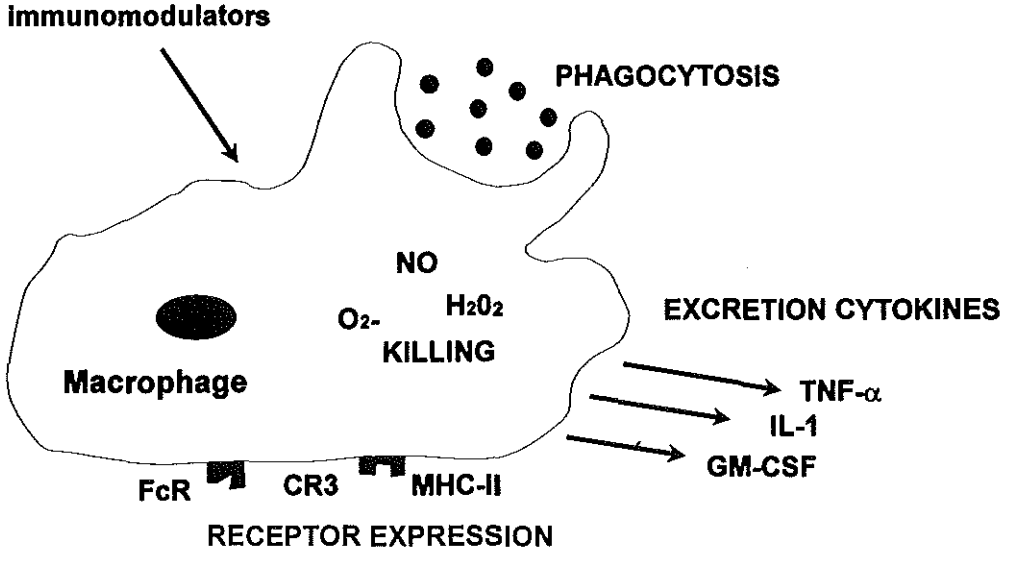

Figure 1. Schematic representation of direct macrophage activation by immunomodulators such as muramyl peptides or interferon-γ. As discussed in the text exposure of macrophages in culture to these immunodulators results in increased activity of these cells, resulting most often in an augmented antimicrobial activity.

macrophage phagocytic capacity towards *Staphyloccocus aureus* (136), or even a negative effect on uptake and killing of *P. aeruginosa* and *S. aureus* by macrophages (119). Culture of human monocytes in the presence of IFN–γ enhanced the capacity to produce superoxide anion. However, phagocytosis of *P. aeruginosa* was substantially depressed in a dose-dependent fashion (119). These findings were supported by in vivo incubation of resident or exsudate peritoneal macrophages with intraperitoneally injected IFN–γ. Addition of IFN–γ did not result in increased in vitro phagocytosis of *Salmonella typhimurium* as compared with untreated mice (30). Similar results were obtained after 18 h of in vitro incubation of resident or exsudate peritoneal macrophages with IFN–γ.

Table 2 In vitro and in vivo studies on antimicrobial enhancing activity of muramyl dipeptide (MDP), muramyl tripeptide phosphatidyl ethanolamine (MTPPE) and IFN–γ, in the free or liposome-encapsulated (LE) form to Gram-negative septicemia causing bacteria.

Micro-organism	Immunomodulator	Immunocompromising factor	Model	Effect[a]	Refs
Escherichia coli	MDP	polymicrobial i.p infection	mouse	+	67
	MDP	obstructive jaundice	rat	0	68
	MDP	obstructive jaundice	mouse	+/0	69
	MDP	-	in vitro	+	70
	MDP	-	mouse	+	71
	MDP	-	mouse	+	72
	MDP	-	mouse	+	73
	MDP	malnourished	mouse	+	74
	MDP	-	rat	+	75
	MDP-Lys (L18)	-	mouse	+	76
	MDP-Ala (L18)	-	mouse	+	77
	MDP-Ala (L18)	-	in vitro	+	77
	MDP-Ala (L18)	-	in vitro	+	78
	LE-MDP	obstructive jaundice	rat	+	68
	LE-MTPPE	obstructive jaundice	rat	+	79
	IFN–γ	malnourished	mouse	+	74
	IFN–γ	-	rat	+	80
	IFN–γ	hyporesponsive to LPS	mouse	+	81
	IFN–γ	-	in vitro	+	82
	IFN–γ	trauma by burns	mouse	+	83
	IFN-γ	hemorrhagic shock	rat	+	84
Klebsiella pneumoniae	MDP	-	mouse	+	72
	MDP	-	mouse	+	73
	MDP	shock	mouse	+	85
	MDP	-	mouse	+	86
	MDP	-	mouse	+	87
	MDP	-	mouse	+	88
	MDP	Cobra venom factor	mouse	+	89
	MDP	-	mouse	+	90
	MDP	starvation	mouse	+	91
	MDP	Cyclophosphamide	mouse	+	92
	MDP	Hydrocortisone	mouse	0	92
	MDP	malnourished	mouse	+	93
	MDP	neonatal	mouse	+	94
	MDP	neonatal	mouse	+	95
	MDP	-	mouse	+	96
	MDP	-	mouse	+	97
	MDP	-	mouse	+	98
	MDP	-	mouse	+	99
	MDP	hyperferremia	mouse	+	100
	MDP-Ala (L18)	-	mouse	0	77
	MDP-Ala (L18)	-	in vitro	+	77
	MDP-Ala	-	ex vivo/in vitro	+	78
	MDP-Lys (L18)	-	mouse	+	76
	MDP-Lys (L18)	-	mouse	0	101
	MTPPE	-	mouse	+	107, 103
	LE-MTPPE	-	mouse	+	102, 103, 104
	LE-MTPPE	Cyclophosphamide	mouse	+	104

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	LE-MTPPE	-	mouse	+	105
	LE-MTPPE	-	in vitro	-	105
	IFN–γ	abdominal trauma	mouse	+	106
	IFN–γ	-	mouse	+	107
	IFN–γ	-	mouse	+	108
	IFN–γ	burn wound infection	mouse	+	109
	IFN–γ	-	in vitro	+	110
	IFN–γ	-	mouse	+	110
	LE-IFN–γ	-	mouse	+	105
	LE-IFN–γ	-	in vitro	0	105
	LE-MTPPE/IFN–γ	-	mouse	+	105
	LE-MTPPE/IFN–γ	-	in vitro	0	105
Pseudomonas aeruginosa	MDP	-	mouse	+	72
	MDP	-	mouse	+	73
	MDP	-	mouse	+	111
	MDP	-	mouse	+	112
	MDP	-	mouse	+	113
	MDP	trauma by burns	guinea pig	0	114
	MDP	-	mouse	+	115
	MDP-Ala (L18)	-	mouse	+	77
	MDP-Lys (L18)	-	mouse	+	76
	MDP-Lys (L18)	Cortison acetate	guinea pig	+	116
	MDP-Lys (L18)	-	in vitro	+	117
	IFN–γ	-	in vitro	+	14
	IFN–γ	burn wound infection	mouse	0	109
	IFN–γ	trauma by burns	mouse	+	118
	IFN–γ	-	in vitro	-	119
Salmonella enteritidis	MDP	myeloperoxidase def. (beige)	mouse	-	120
	MDP	CBA/N (xid)	mouse	+	121
	MDP-Lys (L18)	X-linked immunodeficiency	mouse	+	122
	LE-MDP	CBA/N (xid)	mouse	+	121
	IFN–γ	-	mouse	+	41
	IFN–γ	hypophysectomy	rat	+	123
	IFN–γ	beige / Chediak-Higashi	mouse	+	124
	IFN–γ	-	in vitro	+	125
	IFN–γ	-	in vitro	0	126
Salmonella typhimurium	MDP	CBA/N (xid)	mouse	+	121
	LE-MDP	CBA/N (xid)	mouse	+	121
	IFN–γ	-	mouse/ex vivo	-	30
	IFN–γ	-	in vitro	-	30
	IFN–γ	-	in vitro	-	31
	IFN–γ	-	mouse	+	41
	IFN–γ	-	mouse	+	124
	IFN–γ	nude	mouse	+	124
	IFN–γ	infant	mouse	+	127
	IFN–γ	-	in vitro	+/0	128
	IFN–γ	-	mouse	0	128
	IFN–γ	-	in vitro	+	129
	IFN–γ	-	in vitro	+	130
	IFN–γ	-	in vitro	0	131
	IFN–γ	-	mouse	+	132
	IFN–γ	melanoma	human/ex vivo	+	133
	IFN–γ	-	in vitro	+	134

a Administration of the immunmodulators in vivo or exposure of cell cultures in vitro had a positive (+) or negative (-) effect on the antimicrobial activity, or had no effect at all (0).

In contrast, others found that peritoneal macrophages incubated with IFN–γ for 12 h exhibited enhanced bactericidal activity against *S. typhimurium*, which seemed to be ROI-independent (130). It was also shown that, although IFN–γ treatment enhanced superoxide anion production and intracellular killing of *Salmonella enteritidis* by peritoneal macrophages, of oxygen intermediates scavengers such as superoxide dismutase

and catalase had no effect on at all (126). These results suggest that increased generation of ROI may not be primarily responsible for the observed ability to inhibit intracellular growth of bacteria. Moreover, we observed that peritoneal macrophages exposed to IFN–γ or MTPPE in free form (unpublished observation) or liposome-encapsulated (LE-IFN–γ, LE-MTPPE or (LE-MTPPE/IFN–γ)) resulted in increased production of both ROI and RNI. However, peritoneal macrophages did not exhibit increased phagocytic activity towards *Klebsiella pneumoniae* (105). In contrast, it was demonstrated that MDP-lys(L18), an MDP analog, stimulated alveolar macrophages to phagocytize *P. aeruginosa* (117). Also peritoneal macrophages isolated form mice treated with MDP showed marked augmentation of phagocytosis of *Escherichia coli* in vitro (70). These results clearly show that in vitro exposure of macrophages to IFN–γ or MDP derivatives results in a heterogenous response with respect to bacterial killing. Although production of ROI and RNI is often seen, their role in bacterial killing is still unclear. The discrepancy found is probably not only due to the different bacteria used in these experiments but also depends on macrophage culture purity and condition. We claim from our results that, although in vitro priming of macrophages with immunomodulators results in enhanced metabolic activity, significant antibacterial activity, as seen in vivo, is most likely the result of the activation or participation of other cells apart from macrophages.

2.2. IN VIVO STUDIES

MDP or MDP derivatives, as well as IFN–γ have been shown to stimulate host resistance of mice against *K. pneumoniae* infection in several models. Host resistance could be enhanced by prophylactic intravenous or subcutaneous administration of muramyl peptides to intravenous and intramuscular infections with *K. pneumoniae* (85, 87, 94, 104). Parant et al. demonstrated that also intragastric administration of MDP in mice infected with *K. pneumoniae* resulted in enhanced resistance when administered 7 days before challenge (95). It was shown that orally administered MDP is active against intramuscular infection with *K. pneumoniae* (87): oral administration of 2000 μg MDP 24 h before intramuscular challenge with *K. pneumoniae* improved survival to 52%, against 17% in controls. However, it was shown in that same model that subcutaneous or intravenous administration of 100 μg MDP already resulted in 58% and 63% survival respectively, compared with 21% and 10% survival in the placebo-treated controls. Protective activity of MDP-lys(L18) was demonstrated against *E. coli* infections and to a lesser extent against *P. aeruginosa*. However, only little activity was found towards a *K. pneumoniae* infection. In this study also the oral route of administration appeared effective. A decrease in bacterial survival was observed in blood and organs of *E. coli*-infected mice. (76). Osada et al demonstrated that MDP-lys is highly active against *E. coli* irrespective of the administration route, but especially subcutaneous prophylactic injections with MDP-lys almost completely prevented *E. coli* bacteremia (77). As a contrast orally administered MDP analogs were not active against intraperitoneal *P. aeruginosa* infection, whereas these compounds injected by intravenously, intraperitoneally or subcutanaously route protected mice from the infection (10). Moreover, there was a significant reduction in mortality in mice intraperitoneally infected with *E. coli* and treated with MDP compared to controls (67), which was shown by others to correlate with

increased white blood cell count (75). MDP analogs administered intraperitoneally one day before infection with *P. aeruginosa* enhanced nonspecific host resistance (112). A significant reduction in *E. coli* bacteremia was observed in animals treated with a combination of MDP and clindamycin when compared to animals receiving placebo or either agent alone, indicating that immunomodulation might be beneficial in initially failing antibiotic treatment (71). However, subcutaneous MDP pretreatment failed to enhance cytotoxic activity of the MPS towards intravenous infection with *E. coli*, which is most likely due to the intravenous route of infection. (69)

As the macrophage is considered the primary target for this agent, it is plausible that intravenous administration of immunomodulators is much more potent than oral administration. Although good results are obtained with prophylactically administered MDP, mice infected intramuscularly could even be protected by intravenous administration of MDP 1h after infection (87).

Although good results are obtained in immunocompetent animals, a clinically more relevant option is the treatment of immunodeficient hosts. As stated before, due to the lack of sufficient host defense, especially this group of patients is prone to severe infections, with often a bad prognosis. Important therefore is the capacity of immunomodulators to enhance host resistance adequately in immunocompromised host. MDP was shown to enhance survival from subcutaneous infection with *K. pneumoniae* in 7-day old newborn mice, this in contradiction with LPS treatment (94). LPS, although a strong enhancer of the MPS clearance capacity was unable to increase survival in the *K. pneumoniae*-infected neonates. Also oral administration of MDP derivative: MDP-lys showed protective effect in newborn mice with a subcutaneous infection. Little is known concerning neonates non-specific host defense. However, some agents capable of inducing protective resistance in adults (like LPS) are devoid of activity in neonates (ref). This indicates that MDP does not only affect macrophages directly, but must also have other activities, which are absent in LPS. It is claimed by the same authors that MDP is capable of enhancing host defense to a *K. pneumoniae* infection in thymectomized, irradiated, and bone marrow reconstituted mice (137). Galland et al and Polk et al showed that *K. pneumoniae*-infected wounds can be treated to some extent with MDP in immunocompromised mice (91-93, 97, 98). Survival in mice starved for 48 h and treated prophylactically with 500 μg MDP before intramuscular infection with *K. pneumoniae* was increased to approximately 90% compared with 40% in the controls (91). MDP treatment resulted also in lower local and systemic bacterial spread and increased survival in mice immunosuppressed by cyclophosphamide (92). However, immunosuppression with hydrocortisone was shown to have a deleterious effect in this wound infection model on host defense following activation by MDP. Administration of MDP prior to inoculation of burn wounds with *P. aeruginosa* had no beneficial effect on survival in mice. (114). These results indicate that although host defense can be augmented towards infections in immunocompetent hosts, less favourable effects are observed in the immunocompromised host.

Hershman and colleagues demonstrated that like muramyl peptides also administration of IFN-γ can augment host resistance to a *K. pneumoniae* infection. In a clinically relevant model, mice were infected intraperitoneally with *E. coli* and underwent immediate surgical laparotomy, after which mice were secondarily infected intramuscularly with *K. pneumoniae* (106). Mice receiving a daily subcutaneous dose of 7500 U IFN-γ for 6 days experienced a 2-fold increased survival compared with controls

(63% and 35%, respectively). Prophylactic administration of IFN–γ to mice receiving *K. pneumoniae* intramuscularly or as a wound infection resulted in significant increase in survival compared with controls (106-108, 138). In this model also therapeutically administered IFN–γ (1 h after intramuscular challenge) resulted in significantly augmented host defense (138). Mice treated prophylactically subcutaneously with IFN–γ survived burn wound infections with *K. pneumoniae* longer than controls. However, no beneficial effect was seen when these mice were infected with *P. auruginosa*, which correlates with results obtained with MDP in burn wounds as discussed above (109, 114).

The wound infection model allows to study whether IFN–γ treatment of infections is a local phenomenon, or a systemic effect. Mice infected in the right hind leg and receiving IFN–γ subcutaneously in the same leg showed the same improved survival compared with mice treated with IFN–γ in the other leg, indicating a systemic activity of host defense by IFN–γ (107). These results indicate that host defense activation by IFN–γ is probably mediated by macrophages not necessary located at the site of infection, but resulting from a general strengthening of host defense.

Indirectly, the beneficial effect of IFN–γ in infection is shown by depletion of IFN–γ in vivo. Depletion of IFN–γ decreased resistance of mice infected with *S. typhimurium* resulting in death, indicating an important role for endogenous IFN–γ in host defense towards septicemia (139). However, in vivo depletion of IFN–γ demonstrated that IFN–γ is not required for clearance of *S. typhimurium* from the circulation (132). Moreover, anti-IFN–γ given either 1 h before or 30 min after injection of *E. coli* increased survival of mice from 28% to 83% at 24 h after challenge (140). These results indicate that IFN–γ can also aggravate infection, probably by promoting shock resulting from septicemia. The mechanism behind augmentation of host defense to *K. pneumoniae* infection will be discussed below. Studies in our laboratory on immunomodulation in mice demonstrated that depletion of tissue macrophages completely abrogated host defense stimulation (103).

The recognition of macrophages as target cell in vivo for immunomodulators and key cells in non-specific activation of host defense, favors the attempt to target immunomodulators to macrophages. One possibility to do so is by using liposomes.

3. LIPOSOMES CONTAINING IMMUNOMODULATORS

Although significant macrophage activation occurs after exposure to immunomodulators in vitro, in vivo results are often quite disappointing. Actually this should not be surprising. In vivo experiments are complicated by several factors which include pharmacokinetically dictated effects (short half life, dilution, lack of significant localization at site of interest, serum protein binding and degradation). But also lack of synergy and concomitant toxicity including sensitization are important.

To overcome some of these disadvantages liposomes may be used as carriers. Liposomes are microscopic vesicles consisting of one or more lipid bilayers surrounding an internal aqueous compartment. By encapsulating agents in liposomes life-time in the body is prolonged, high concentrations at specific sites can be reached, and by co-encapsulation of agents synergy in vivo can be assured. Also, toxicity of the encapsulated compound may be reduced and immunologic reaction may be prevented. In addition,

liposomes avoid serum protein binding and dilution (141). Liposomes are biodegradable, and non-immunogenic if composed of natural phospholipids. A variety of agents can be entrapped in liposomes: hydrophobic agents with high efficiency in the lipid bilayers, and hydrophilic agents in the inner aqueous space.

As macrophage are believed to be the most important target cells for immunomodulation the use of classical liposomes, which tend to localize in large numbers in these cells, is quite obvious. Classical liposomes are relatively large (ranging from 1 to 20 μm in diameter) and do not have features which diminish uptake by the MPS, in contrast to the newer generation liposomes such as the MPS-avoiding Stealth® liposomes.

Tendency of classical liposomes to rapidly localize in MPS cells, primarily macrophages residing in liver and spleen (142), results in an augmented accumulation of encapsulated agent in macrophages. Phospholipid composition, charge and size have a strong influence on uptake and degradation of liposomes by macrophages. Specifically, inclusion of negatively charged phospholipid such as phosphatidylserine (PS) in liposomes consisting of phosphatidylcholine (PC) enhances binding to and phagocytosis by macrophages, whereas uncharged liposomes composed exclusively of PC are less efficiently taken up by macrophages (141). Pharmacokinetics in rats show that 30 min after intraveneous injection, MTPPE encapsulated in liposomes (PC:PS, molar ratio 7:3) is mainly located in liver (55% of injected dose), lung (19%), and spleen (15%) (143). Six hours after injection LE-MTPPE is mainly located in liver (40%) and spleen (17%), whereas a decrease in pulmonary uptake (3.4%) is observed. Similarly, in mice LE-MTPPE was preferentially taken up by liver and spleen (32% and 17% respectively after 60 min). Localization in lung however only reached 8.4% of injected dose after 5 min, declining rapidly below 5% after 60 min (142).

Good targeting of liposomes to macrophages is shown. However, association of the encapsulated agents with liposomes has to be long enough to ensure their delivery to the site of interest. Initial studies with liposomes composed of PC and PS (molar ratio, 7:3) encapsulating MDP demonstrated that MDP was poorly retained within liposomes after their preparation (50% released in 5 h at 37°C) (144). The lipophilic derivative MTPPE however, has been shown not only to associate more efficiently with liposomes (93%), but also to be retained longer by liposomes (7). The total extent of leakage was shown to range from 0.8% to 9% depending on dose and experimental setting (145). Encapsulation of muramyl peptide derivatives and IFN-γ may thus prolong life-time of these agents in the body; 90% of free MDP was cleared form the body and excreted in the urine in 2 h, whereas excretion rate of LE-MDP was only 3-6% per h (13). For LE-MTPPE, plasma levels of free MTPPE is very low compared with free MTPPE. In rats and dogs an intravenous dose of 0.2 and 0.1 mg/kg LE-MTPPE was cleared from the blood compartment for 96% and 100%, respectively at 10 min after injection (145). Rapid clearance of LE-MTPPE from circulation is mediated by tissue macrophages, and not via excretion in urine. Intact liposomes can be observed in macrophages for several days (141, 146). These results indicate that LE-MTPPE forms a depot of immunomodulatory material within the phagolysosomal system of macrophages and that considerable time (up to days) is needed to degrade liposomes and subsequently release incorporated muramyl peptide. Macrophages exposed to LE-MDP or LE-MTPPE are tumoricidally active for a longer period compared to cells exposed to free MDP or free MTPPE (144).

In mice bearing B16/BL6 melanoma metastases LE-MTPPE was shown to be 10 times more potent than MTPPE in the free form (147). In studies done by others LE-MDP appeared to be 4000 times more active than free MDP in rendering macrophages tumoricidal in vivo (13). On a molar basis MTPPE is approximately 2.5-fold more efficient than MDP in eliciting comparable levels of macrophage tumoricidal activity (13). It was shown that free MTPPE is 40-fold more potent than MDP to induce tumoricidal activity in a human monocyte system, due to proportionally greater uptake of MTPPE by MPS cells (7). A further increase of MTPPE activity when encapsulated into liposomes was obtained in vivo (102, 148). There is thus a large discrepancy between activity of MDP and MTPPE in vivo, which is probably due to hydrophobic nature of MTPPE. MTPPE spontaneously forms micelles in water and is therefore more readily recognized by macrophages than MDP. This effect is likewise seen with liposomes. However, there is only a small increase in activity by liposomal encapsulation of MTPPE. The better immunomodulatory effects obtained with LE-MTPPE compared with LE-MDP might be explained by the better association of MTPPE with the liposomes compared with MDP, resulting in a higher immunomodulator-lipid ratio, and a prolonged association of MTPPE with the liposome.

The use of IFN–γ for immunotherapy is limited by rapid clearance of this cytokine from circulation, and by toxicity seen with high dosage regimens (149-151). Free IFN–γ has a serum half-life time in humans of approximately 20 min, and is degraded and secreted from the body. Liposome-encapsulation of IFN–γ (LE-IFN–γ) increases its half-life time and, as will be discussed below, also increases its ability to stimulate the host defense.

The increased activity of LE-MTPPE and LE-IFN–γ over free immunomodulators was shown in an in vivo infection model using *Listeria monocytogenes* in our laboratory. Encapsulation of MTPPE or IFN–γ increased their efficacy 33- and 66-fold respectively in mice infected with *L. monocytogenes* (148). An increased activity of LE-MTPPE over free MTPPE was also shown in a *K. pneumoniae* infection model as is discussed in the next section (102). Moreover, prophylactic treatment with MDP analogs significantly enhanced host resistance in mice towards lethal challenge with *S. typhimurium* and *S. enteritidis*. Encapsulation of these analogs in liposomes enhanced their anti-infectious activity 10- to 15-fold (120, 144). It is tempting to speculate that increase in host defense results from the enhanced clearance after immunomodulation.

Both MTPPE and IFN–γ induce undesirable side-effects such as fever, weight loss, liver and kidney disfunction. MTPPE also induces histopathologic changes in arteries. Liposomal encapsulation decreased toxicity of MTPPE approximately 10-fold (147). Reduction of toxicity of IFN–γ by liposomal encapsulation has also been demonstrated (52, 53).

Together, these results demonstrate that liposomal encapsulation will reduce toxicity of these agents probably by reducing undesirable localization to sensitive sites (site avoiding delivery), but also increases localization at the site of interest, macrophage (site specific delivery). This means that lower dosages can be applied, and better results may be obtained in vivo when these immunomodulators are encapsulated into liposomes. Importantly, it was observed that in the *K. pneumoniae* infection models antimicrobial effects were maximal if immunomodulators were administered 24 h or more before infection (95, 102, 104). Prophylactically administered LE-MTPPE as a single dose, or repeatedly for five dosages resulted in activation of host defense and improved survival

of mice from a *K. pneumoniae* septicemia (105). These results indicate that prophylactic administration is the prefered dosing regimen for such agents.

It might be expected that administration of high dosages of liposomes results in saturation of the MPS cells, and by doing so reduces host defense. Phagocytic capacity of the MPS for bacteria might consequently be reduced. However, we found that 5-fold repeated injection in seven days of a high dose of placebo liposomes (125 mg lipid per kg per dose) in mice did not negatively affect clearance capacity of the MPS (105).

The possibility of co-encapsulation of above mentioned agents into liposomes also provides an important tool for drug delivery in vivo. Synergy between immunomodulating agents can be obtained in vitro. However, in vivo synergy is questionable since in vivo simultaneous exposure of macrophages to multiple immunomodulators after intravenous administration is expected to be minimal due to wide differences in their pharmacokinetic behaviour. With agents co-encapsulated in liposomes, simultaneous delivery of agents to macrophages is guaranteed. Synergy between MTPPE and IFN–γ in the free form was shown in vitro using *L. monocytogenes*-infected peritoneal macrophages (148). Striking synergy between muramylpeptides and macrophage-activating cytokine (i.e. IFN–γ) was also shown in several tumor models (7) in vitro and in vivo. Co-encapsulation of MTPPE and IFN–γ also improved survival of mice suffering from a *K. pneumoniae* septicemia compared with these agents in the free from, as will be discussed in the next section.

4. LIPOSOME-ENCAPSULATED MTPPE AND IFN–γ IN *KLEBSIELLA PNEUMONIAE* SEPTICEMIA

We demonstrated that liposomal encapsulation of MTPPE enhanced its activity compared to free MTPPE in a *K. pneumoniae* septicemia model in which bacteria are injected intravenously. A single dose of 25 μg LE-MTPPE resulted in 100% survival compared to 50% survival in mice treated with 25 μg free MTPPE 24 h before intravenous challenge (102). In a murine model mimicking a naturally acquired septicemia with *K. pneumoniae* the effect of MTPPE and IFN–γ on host defense was studied. In this model bacteria are injected intraperitoneally, allowing the bacteria to multiply and appear in the blood, resulting in a septicemia followed by death of all animals within 5 days after challenge. It was shown in our laboratory that prophylactic treatment with a single dose of 50 μg MTPPE or 25 μg LE-MTPPE resulted in 55% and 40% survival, respectively. However, repeated prophylactic administration of LE-MTPPE (4 dosages of 3.1 μg daily), resulted in a survival of 75%. These findings indicate that MPS cells do not become refractory to further activation, but that more MPS cells are activated, or that activation of the cells becomes higher after repeated immunomodulator administration. Beneficial effect of multiple treatment was also shown with a MDP analog in an intraperitoneal infection model with *P. aeruginosa*. Optimal treatment of 80 mg/kg of norMDP per day given intraperitoneally before infection increased survival from 45% in control mice or mice treated with a single dose of norMDP, to 90 % in mice receiving 4 dosages (115). These investigators obtained similar results with *Candida albicans* infection.

In the *K. pneumoniae* septicemia model utilized in our laboratory the effect of LE-IFN–γ and the liposome-encapsuleted combination of IFN–γ with MTPPE was studied. Intravenous injection of a single dose of LE-IFN–γ 24 h before intraperitoneal infection

resulted in 15% survival (105). In parallel with results obtained with LE-MTPPE multiple dosages of LE-IFN–γ could further increase survival of mice to 65%. Moreover, combination of MTPPE together with IFN–γ by co-encapsulation in liposomes further enhanced host resistance: 100% survival was obtained (105). The effect of co-encapsulated MTPPE and IFN–γ on host defense was additive, suggesting that these two immunomodulators have their effect independent of each other.

4.1. MECHANISMS

From studies by others and us in vitro it can be concluded that both muramyl peptides and derivatives (like MDP and MTPPE) and IFN–γ are potent stimulators of macrophage function. Exposure of macrophages to these agents results in an enhanced metabolic activity, excretion of ROI and RNI, production of important host defense activating monokines, and an increased antimicrobial activity of the cells. However, in vivo studies do not always support these findings. Although in vitro increased killing of the intracellular bacterium *L. monocytogenes* was found in our laboratory and by others after administration of MDP, MTPPE or LE-MTPPE, it was also found that in vivo MDP had no activity against *L. monocytogenes*. Possible reasons for this disparity might be due to dosage, treatment schedule, challenge level, virulence of the pathogen used, as well as the formulation of the immunomodulator (i.e. liposomal), rather than the mechanism by which host defense is activated.

Studies with macrophages exposed to LE-MTPPE, LE-IFN–γ or LE-MTPPE/IFN–γ in vitro demonstrated an enhanced production of ROI or RNI when stimulated with heat-killed Gram-negative bacteria (105). However, an increased antibacterial activity to *K. pneumoniae* could not be found in vitro when isolated macrophages were exposed to above mentioned immunomodulators. Uptake of *K. pneumoniae* as well as killing of intracellular microorganisms by macrophages exposed to immunomodulators was not different from controls. Moreover, in vitro results also indicated that the observed increase in survival in immunomodulator-treated mice can not be explained solely by increased activity of the tissue macrophages themselves. We speculate that in this infection model tissue macrophages still serve as key cells in host activation by immunomodulators, but that upon macrophage stimulation also modulation of other parts of host defense system is involved. It has been demonstrated that administration of MDP or MTPPE (free or liposome-encapsulated) resulted in an increased blood clearance capacity of MPS cells (85, 94, 103). Also, in an infection with *P. aeruginosa* increased protection after treatment with MDP analogs was found to correlate with degree of clearance of particles form the blood by the MPS (152). It may thus be hypothesized that increased survival from Gram-negative infection in mice induced by these immunomodulators resulted in the first place from an augmented phagocytic activity of tissue macrophages, and hence an increased clearance of bacteria from blood. These speculations are supported by findings with another septicemia-causing microorganism. Mice pretreated with IFN–γ before infection with *S. typhimurium* showed significantly increased survival. Declined bacterial count in liver and spleen suggest that IFN–γ treatment may have resulted in more efficient killing of bacteria by phagocytic cells (124). These results are in agreement with prophylactic intraperitoneal treatment (6 h before challenge) of mice infected intraperitoneally with *S.*

enteritidis (125). A diminished bacterial growth in peritoneal cavity, liver and spleen, which was speculated to be a result of increased bacterial clearance rather than prevention of systemic spread.

Others demonstrated in a wound infection model that locally the access of bacteria to the bloodstream is restricted after treatment with MDP (98). In that mechanism according to Polk et al. containment is very important, which is accentuated by MDP. Escape of bacteria from the site of infection to the bloodstream was much higher in control mice compared with MDP treated mice. Local growth was not affected significantly by MDP treatment. However, regional lymphatic filtration was markedly improved (100-fold fewer bacteria progressed from iliac nodes to blood). Polk et al claim that MPS cells are not significantly activated by MDP, because increase of bacterial concentration in liver coincided with an increase in the degree of bacteremia. This is also true for starved (malnourished) mice or mice treated with cyclophosphamide with a wound infection: enhanced containment resulting in less bacteria in blood (98). However, when bacteria do progress to blood, lymphatic filtration cannot explain the improved resistance induced by immunomodulators. Increased phagocytic activity of MPS cells on the other hand is also not a likely explanation. It was shown in our laboratory that exposure of peritoneal macrophages to liposomal formulations of MTPPE, IFN-γ or these agents combined in vitro did not increase uptake or killing of *K. pneumoniae*. The discrepancy between adequate activation of macrophages in vitro resulting in enhanced production of antimicrobial agents and disappointing results obtained in vivo in part might be explained by enhanced macrophage cell numbers, other than increased cell function.

We found a highly significant increase in the number of granulocytes and monocytes circulating in the blood after treatment with free MTPPE or LE- MTPPE (102, ten Hagen et al., manuscript submitted for publication; Chapter 4). Intravenous infection of mice with *K. pneumoniae* normally resulted in a sharp decline in the number of circulating leukocytes. However, prophylactic administration of free or liposomal MTPPE resulted in a stabilization or even a slight increase in leukocyte number. In a porcine pneumococcal septicemia model MTPPE pretreatment resulted in less pronounced leukocytopenia, with a nadir of 6700 leukocytes per μl compared with 4100 in controls (153).

To get more insight in the effects of LE-MTPPE/IFN-γ on the status of MPS cells we studied its composition in more detail in our laboratory. We found that LE-MTPPE/IFN-γ resulted in the first place in much higher hemopoietic cell numbers in liver and spleen (ten Hagen et al., manuscript submitted for publication). Especially myeloid cell numbers (monocytes and macrophages) were increased in these organs, whereas strongly increased erythropoiesis was also observed in the spleen. Secondly, treatment with LE-MTPPE/IFN-γ induced a shift in bone marrow hemopoiesis towards generation of myeloid cells, whereas erythropoiesis declined. These results indicate that immunomodulation will result in a dramatic increase in the number of MPS cells, which may help explain the increased phagocytic capacity of this system. It is tempting to speculate that more cells doing a better job (quantity) is the explanation in vivo rather than the same cells doing a better job (quality). Together these results suggest **1)** increased recruitment of macrophages and granulocytes from bone marrow, **2)** local proliferation of myeloid cells, and **3)** augmented hemopoiesis in bone marrow, accounting for the observed host defense improvement, rather than enhanced phagocytic activity of the single MPS cell. In figure 2 a simplified

scheme is given showing the proposed mechanism of host defense activation by immunomodulators which is thought to be controled by macrophages. Another striking observation is the dramatic augmented erythroblast cell number in the spleen after immunomodulation. It might be that the often observed anemia accompanying sepsis may also be prevented by the LE-MTPPE/IFN–γ treatment.

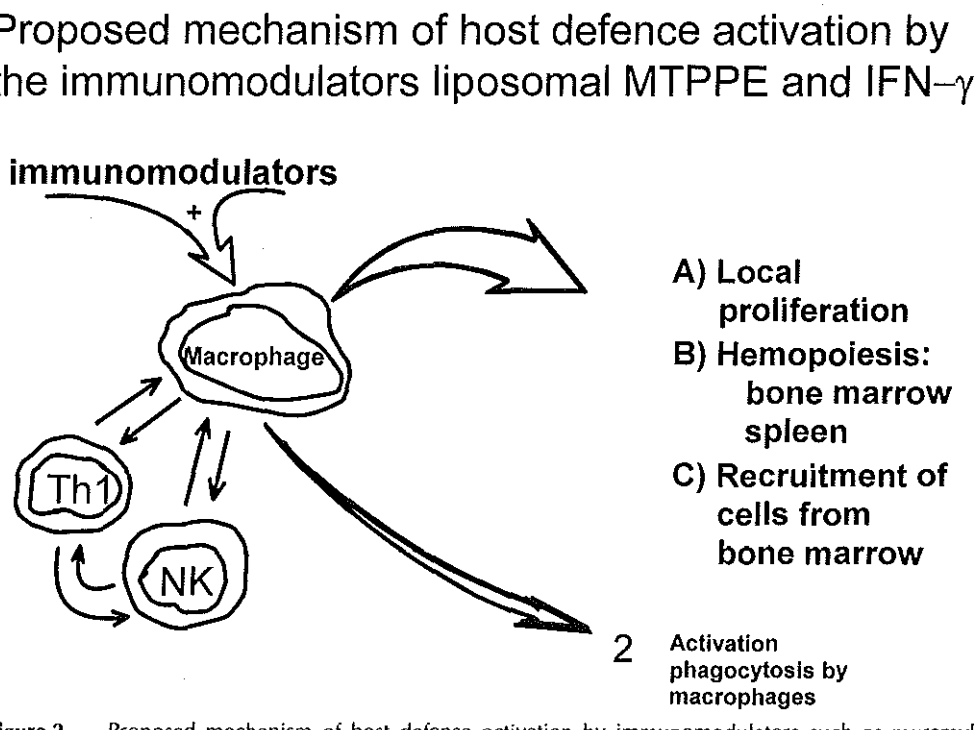


Figure 2. Proposed mechanism of host defense activation by immunomodulators such as muramyl peptides or interferon–γ, which is thought to be controlled by macrophages. As described in the text immunomodulation in vivo results in activation of macrophages, which involves also other cells of the immune-system such as T and NK cells. This activation results in local proliferation of macrophages, increased hemopoiesis, and recruitment of monocytes and granulocytes from bone marrow to the periphery. These activities are thought to be of major importance for host defense activation to infection. Direct antimicrobial activation of macrophages is thought to be of only minor importance.

Muramyl peptides (MDP, MTPPE) and IFN–γ do have a direct effect on macrophages. It was shown that also intracellular localization of agents following administration in liposome-encapsulated form resulted in enhanced antimicrobial activity of the cells (141). For both muramyl peptides and IFN–γ intracellular receptors were found (154-156). As stated before, administration of hydrocortisone abrogated host defense activation completely, whereas cyclophosphamide treated animals could still be activated to a certain extent. Steroids exert major immunosuppressive and anti-inflammatory effects in many organ systems, impair antibody production and cell mediated immunity. T cell migration is abrogated, monocyte migration and extravasation is impaired, production of

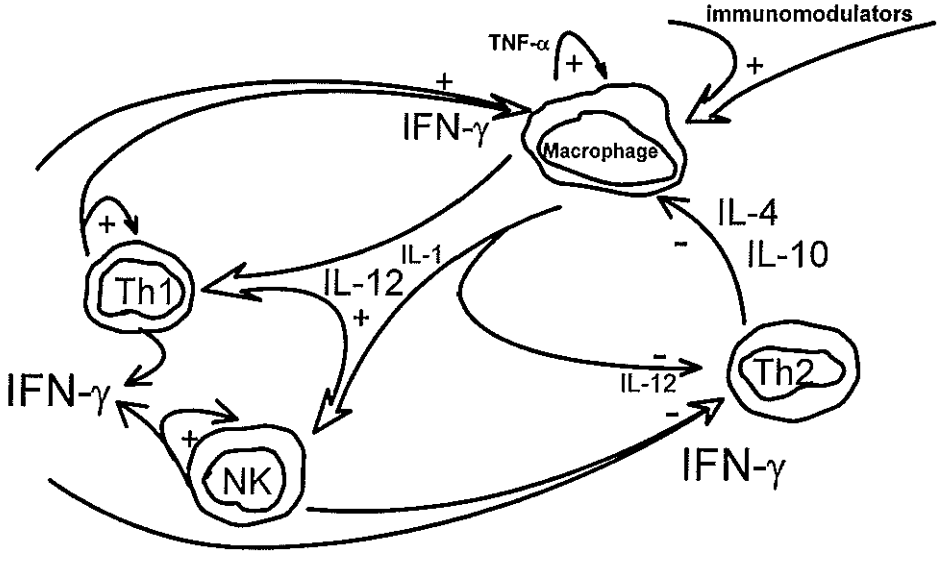
cytokines by lymphocytes and mononuclear cells is blocked, polymorphonuclear inflammatory response reduced, and lymphocyte, monocyte, eosinophil, and basophil counts in peripheral blood were all decreased after steroid therapy (157). Cyclophosphamide induces bone marrow suppression resulting in severe granulocytopenia, as well as B cell depletion. However, macrophages are not affected and even increased DTH can be observed (158). The differences in activity of these two immunosuppressive agents would indicate that maintained macrophage function in cyclophosphamide treated animals is crucial for effective immunomodulation.

Upon stimulation macrophages produce many different cytokines (i.e IL-1, TNF–α, IL-12 etc). Especially IL-1 and IL-12 have a stimulating effect on T cells and NK cells that respond with production of IFN–γ, which in turn has a stimulating effect on macrophages. Studies with MDP, MTPPE or IFN–γ demonstrated increased colony stimulating activity in serum after treatment. TNF–α production by macrophages is also strongly increased (159-161), and is known to have stimulating activities on growth and development of lymphoid tissues. IFN–γ stimulates production of CSF and IL-6, known to have strong hemopoietic activities. A very schematic representation of the possible cytokine network induced by the immunomodulators is given in figure 3, which network is likely to result in above described hemopoietic effects. This mechanism also includes an important role for T cells. It was shown that potentiation of resistance by IFN–γ is also possible in *Leishmania donovani*-infected euthymic mice, but not in nude mice (162). Injections of 10^5 U IFN–γ every second day (7 days) induced killing of *L. donovani* in livers of euthymic mice, but had no effect in nude mice, despite shown activation of peritoneal macrophages in vitro. Transfer of $CD4^+$ or $CD8^+$ T cells permitted nude mice to respond to IFN–γ treatment, which on the other hand could not be compensated with T cell derived cytokines alone. NK cells or NK derived endogenous IFN–γ did not seem to play any apparent role. The antileishmanial effect correlated with a markedly enhanced mononuclear cell recruitment to infected liver foci. It was demonstrated in our laboratory that T cells play a very important role in the LE-MTPPE, LE-IFN–γ or LE-MTPPE/IFN–γ increased host defense to *K. pneumoniae* septicemia (ten Hagen et al., manuscript submitted for publication; Chapter 3). Depletion of $CD4^+$ T cells or $CD8^+$ T cells dramatically inhibited antimicrobial potentiation by immunomodulators. Moreover, blocking of IFN–γ in vivo demonstrated that especially production of endogenous IFN–γ is important in host defense activation by immunomodulators. It was shown that treatment with LE-MTPPE/IFN–γ preferentially induced a Th1 T cell response in spleen. Macrophages in spleen were shown to be significantly activated by this treatment as shown by enhanced expression of MHC class II, FcRI and CR3 receptors. It is tempting to speculate that Th1 T cells , $CD8^+$ cells and NK cells (cells known to produce IFN–γ) play a key role in the cytokine network induced by macrophage targeted immunomodulators.

Next page

Figure 3. Proposed simplified scheme of cytokine network which evolves from in vivo administration of immunomodulators such as muramyl peptides or interferon–γ, and shows interaction of macrophages with T and NK cells. Macrophages are thought to play a central role in host defense activation. Production of monokines such as IL-12 and IL-1 results in activation of Th1 and NK cells. These cells respond with production of IFN–γ which in turn activates macrophages.

Proposed cytokine network of host defence activation by the immunomodulators liposomal MTPPE and IFN–γ



5. FUTURE PROSPECTS

In vivo studies with LE-MTPPE and LE-IFN–γ in animals, as well as clinical trials with LE-MTPPE show that these immunomodulators are relatively safe and can be used repeatedly without saturation of host defense and without cells becoming refractory to treatment (105, 163). With the ongoing problems with severe infections, and the inability of antibiotics to provide adequate therapy in immunodeficient patients, selected patient groups may well benefit from immunomodulation. As is shown above that most promising results can be expected with prophylactic treatment. This means that patient groups would preferably be selected a priori. Especially patients which are likely to acquire immunodeficiency or are about to be rendered immunodeficient in the course of treatment for their underlying disease (e.g. transplantation recipients and patients undergoing major surgery) are potential candidates. Most of these patients will become immunodeficient gradually, giving the opportunity to start administration of immunomodulators before severe host defense breakdown has occurred. Good results can only be anticipated when formulations (for instance liposomal co-encapsulated MTPPE plus IFN–γ) are used in patients, still in possession of their immune system. In these patients immunomodulation may well be tested in combination with antibiotics. Hopefully this combination will give us possibilities to combat infections in these patients with more success.

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REFERENCES

1 Bone RC. 1993. Gram-Negative Sepsis: a Dilemma of Modern Medicine. Clin. Microbiol. Rev. 6:57-68.

2 Bernhishel-Broadbent J., E. E. Camargo, H. S. Jaffe and H. M. Lederman. 1991. Recombinant human interferon-gamma as adjunct therapy for Aspergillus infection in a patient with chronic granulomatous disease. J. Infect. Dis. 163:908-911.

3 Murray HW. 1994. Interferon-Gamma and Host Antimicrobial Defence: Current and future clinical applications. Am. J. Med. 97:459-467.

4 Morrison C. J. and D. A. Stevens. 1989. Enhanced killing of *Blastomyces dermatitidis* by gamma interferon-activated murine peripheral blood polymorphonuclear neutrophils. Int. J. Immunopharmacol. 11:855- 862.

5 Murray H. W. 1989. The activated macrophage and host defense against microbial challenge. Ann. Int. Med. 108:595-608.

6 Murray, H.W. 1992. The interferons, macrophage activation, and host defence against nonviral pathogens. J. Interferon Res. 12:319-322.

7 Dukor P. and G. Schumann. 1987. Modulation of non-specific resistance by MTP-PE In: Majde J. A, ed. Immunopharmacology of infectious diseases. Vaccine adjuvants and modulators of non-specific resistance of infectious diseases. New York: Alan R. Liss Inc., 255-265.

8 Farace F, C. Matiot, M. Brandeley, et al. 1990. Phase-I trial with recombinant interleukin-2 (rIL-2): immune activation by rIL-2 alone or following pretreatment with recombinant interferon-gamma. Clin. Exp. Immunol. 82:194-199.

9 Eppstein D. A., M. A. Van der Pas, E. B. Fraser-Smith, et al. 1986. Liposome-encapsulated muramyl dipeptide analogue enhances nonspecific host immunity. Int. J. Immunotherapy 2:115-126.

10 Fraser-Smith, E.B., D.E. Eppstein, M.A. Larsen, and T.R. Matthews. 1983. Protective effect of a muramyl dipeptide analog encapsulated in or mixed with liposomes against *Candida albicans* infection. Infect. Immun. 39:172-178.

11 Blasi E., S. Farinelli, L. Varesio and F. Bistoni. 1990. Augmentation of GG2EE Macrophage cell line-mediated anti-Candida activity by gamma interferon, tumor necrosis factor, and interleukin-1. Infect. Immun. 58:1073-1077.

12 Brummer, E. and D. A. Stevens. 1989. Candidacidal mechanisms of peritoneal macrophages activated with lymphokines or gamma-interferon. J. Med. Microbiol.28:173-181.

13 Fogler, W.E. and I.J. Fidler. 1984. Modulation of the immune response by muramyl dipeptide. In: Immune Modulation Agents and Their Mechanisms. Fenickel, R.L. and M.A. Chirigos eds. Marcel Dekker, New York, NY. pp 499-512.

14 Kemmerich B., T. H. Rossing and J. E. Pennington. 1987. Comparative oxidatieve microbicidal activity of human blood monocytes and alveolar macrophages and activation by recombinant gamma interferon. Am. Rev. Respir. Dis.136:266-270.

15 Tansho, S., S. Abe, and H. Yamaguchi. 1994. Inhibition of *Candida albicans* growth by murine peritoneal neutrophils and augmentation of the inhibitory activity by bacterial lipopolysaccharide and cytokines. Microbiol. Immunol. 38:379-383.

16 Beaman L. 1987. Fungicidal activation of murine macrophages by recombinant gamma interferon. Infect. Immun. 55:2951-2955.

17 Beaman L. 1991. Effects of recombinant interferon and tumor necrosis factor on in vitro interactions of human nuclear phagocytes with *Coccidioides immitis*. Infect. Immun. 50:4227-4229.

18 Carlin, J.M., and J.B. Weller. 1995. Potentation of interferon-mediated inhibition of Chlamydia infection by interleukin-1in human macrophage cultures. Infect. Immun. 63:1870-1875.

19 Murray H. M., A. M. Granger and F. Teitelbaum. 1991. Gamma interferon-activated human macrophages and *Toxoplasma gondii, Clamydia psittaci* and *Leismania donovani*: Antimicrobial role of limiting intracellular iron. Infect. Immun. 59:4684-4686.

20 Bhardwaj N., T. W. Nash and M. A. Horwitz. 1986. Interferon-gamma-activated human monocytes

inhibit the intracellular multiplication of *Legionella pneumophila*. J. Immunol. 137:2662-2669.

21 Jensen A. W., R. M. Rose, A. S. Wasserman, T. H. Kalb, K. Anton and H. G. Remold. 1987. In vitro activation of the anti bacterial activity of human pulmonary macrophages by recombinant gamma-interferon. Infect. Dis. 155:574-577.

22 Klein T. W., Y. Yamamoto, H. K. Brown and H. Friedman. 1991. Interferon gamma induced resistance to *Legionella pneumophila* in susceptible A/J mouse macrophages. J. Leukoc. Biol. 49:98-103.

23 Nash T. W., D. M. Libby and M. A. Horwitz. 1988. IFN-gamma-activated human alveolar macrophages inhibit the cellular multiplication of *Legionella pneumophila*. J. Immunol. 140:3978-3981.

24 Hadden J. W., E. Arthur, J. R. Sadlik and E. M. Hadden. 1979. The comparative effects of isoprinosine, levamisole, muramyl dipeptide and SM1213 on lymphocyte en macrophage proliferation and activation in vitro. Int. J. Immunopharmacol. 1:17-27.

25 Bakker-Woudenberg I. A. J. M., A. F. Lokerse J. C. Vink-van den Berg and F. H. Roerdink. 1989. Effect of free or liposome-encapsulated MDP on the uptake and intracellular survival of *Listeria monocytogenes* in peritoneal mouse macrophages in vitro. Eur. J. Clin. Microbiol. Infect. Dis. 8:603-609.

26 Chen Y., A. Nakane and T. Minagawa. 1989. Recombinant murine gamma interferon induces enhanced resistance to *Listeria monocytogenes* infection in neonatal mice. Infect. Immun. 57:2345-2349.

27 Kiderlen A. F., S. H. E. Kaufmann and M. L. Lohmann-Matthes. 1984. Prodection of mice against the intracellular bacterium *Listeria monocytogenes* by recombinant immune interferon. Eur. J. Immunol. 14:964-967.

28 Kurtz R. S., K. M. Young and C. J. Czuprynski. 1989. Separate and combined effect of recombinant interleukin-1-alpha and gamma interferon on antibacterial resistance. Infect. Immun. 57:553-558.

29 Nakane A., T. Minagawa, I. Yasudo, Y. Chen and K. Kato. 1988. Prevention by gamma interferon of fatal infection with *Listeria monocytogenes* in mice treated with cyclosporin A. Infect. Immun. 58:2386-2388.

30 Van Dissel J. T., J. J. M. Stikkelbroeck, B. C. Michel, M. T. Van den Barselaar, P. C. J. Leijh and R. van Furth. 1987. Inability of interferon gamma to activate the antibacterial activity of mouse peritoneal macrophages against *Listeria monocytogenes* and *Salmonella typhimurium*. J. Immunol. 139:1673-1678.

31 Van Furth R and J. T. Van Dissel. 1989. Interferon gamma does not enhance the bactericidal activity of murine macrophages. Agents Action 26:158-159.

32 Khor M., D. B. Lowrie and D. A. Mitchison. 1986. Effects of recombinant interferon-gamma and chemotherapy with isoniazid and rifampicin on infections of mouse peritoneal macrophages with *Listeria monocytogenes* and *Mycobacterium microti* in vitro. Br. J. Exp. Pathol. 67:707-717.

33 Peck R. 1989. Gamma interferon induces monocyte killing of Leisteria monocytogenes by an oxygen dependent pathway; Alpha- or beta-interferons by oxygen-independent pathways. J. Leukoc. Biol. 46:434-440.

34 Portnoy D. A., R. D. Schreiber, P. Connelly and L. G. Tilney. 1989. Gamma interferon limits access of *Listeria monocytogenes* to the macrophages cytoplasm. J. Exp. Med. 170:2141-2146.

35 Denis, M., and E.O. Gregg. 1991. Modulation of *Mycobacterium avium* Growth in Murine Macrophages: Reversal of Unresponsiveness to Interferon-Gamma by Indomethacin or Interleukin-4. J. Leukoc. Biol. 46:65-72.

36 Blanchard D. K., M. B. Michelini-Norris and J. Y. Djeu. 1991. Interferon decreases the growth inhibition of *Mycobaterium avium-intracellulare* complex by fresh human monocytes but not by culture-derived macrophages. J. Infect. Dis. 164:152-157.

37 Flesh I. E. A. and S. H. E Kaufmann. 1991. Mechanisms involved in mycobacterial groth inhibition by gamma interferon-activated bone marrow macrophages: role of reactive nitrogen intermediates. Infect. Immun. 59:3213-3218.

38 Douvas G. S., D.L. Looker, A. E. Vatter and A. J. Crowle. 1985. Gamma interferon activates human macrophages to become tumoricidal and leishmanicidal but enhances replication of macrophage-associated mycobacteria. Infect. Immun. 50:1-8.

39 Khor M., D. B. Lowrie, A. R. M. Coates and D. A. Mitchison. 1986. Recombinant interferon gamma

and chemotherapy with isoniazid and rifampicin in experimental murine tuberculosis. Br. J. Exp. Pathol. 67:587-596.

40 Lederer E. 1986. Immunomodulation by muramyl peptides and Trehalose diesters in experimental parasitology. Int. J. Immunotherapy 4:267-278.

41 Ganova L. A, I. V. Fil'chakov, N. Y. Ivanenko and V. S. Zueva. 1988. Production of a factor the bactericidal action of mouse macrophages and its stimulation by interferon. Bull. Exp. Biol. Med. Engl. TR 105:553-555.

42 Sechler J. M. G., H. L. Malech, C. J. White and J. I. Gallin. 1988. Recombinant human gamma intereron reconstitutes defectie phagocyte function in patients with chronic granulomatous disease of childhood. Proc. Natl. Acad. Sci. USA 85:4874-4878.

43 Koff W. C., S. D. Showalter, B. Hampar and I. J. Fidler. 1985. Protection of mice againstn fatal herpes simplex type 2 infection by liposomes containing muramyl tripeptide. Science 228:495-497.

44 Pinto A. J., P. S. Morahan and M. A. Brinton. 1988. Comparative study of various immunomodulators for macrophage and natural killer cell activation and antiviral efficacy against exotic RNA virussus. Int. J. Immunopharmacol. 10:197-209.

45 Masihi K. N., W. Lange, B. Rohde-Schulz and L. Chedid. 1990. Muramyl dipeptide inhibits replication of human immunodeficiency virus in vitro. AIDS Res. Hum. Retroviruses 6:393-399.

46 Chomel J. J., N. Simon-Lavoine, D. Thouvenot, M. Valette, et al. 1988. Prophylactic and therapeutic effects of murabutide OF1 mice infected with influenza A/H3N2 (A/Texas/1/77) Virus. J. Biol. Response Modif. 7:581-586.

47 Dietrich F. M., H. K. Hochkeppel and B. Lukas. 1986. Enhancement of host resistance against virus infections by MTP-PE, a synthetic lipophilic muramyl peptide-I. Increased survival in mice and guinea pigs after single drug administration prior to infection, and the effect of MTP-PE on interferon levels in sera and lungs. Int. J. Immunopharmacol. 8:931-942.

48 Gangemi J. D., M. Nachtigal, D. Barnhart, L. Krech and P. Jani. 1987. Therapeutic efficacy of liposomencapsulated ribavirin and muramyl tripeptide in experimental infection with influenza or herpes simplex virus. J. Infect. Dis. 155:510-517.

49 Masihi K. N., H. Kröger, W. Lange and L. Chedid. 1989. Muramyl peptides confer hepatoprotection against murine viral hepatitis. Int. J. Immunopharmacol. 11:879-886.

50 Kende M., A. Schroit, W. Rill and P. G. Can onico. 1983. Treatment of Rift Valley fever virus-infected swiss mice with liposome encapsulated lipophylic muramyl dipeptide (LE-MDP). ICAAC:abstract 153.

51 Koff W. C. and I. J. Fidler. 1985. The potential use of liposome mediated antiviral therapy. Antiviral Res. 5:179-190.

52 Hockertz S., G. Franke, E. Kniep and M. L. Lohmann-Matthes. 1989. Mouse interferon-gamma in liposomes; pharmacokinetics, organ-distribution, and activation of spleen and liver macrophages in vivo. J. Interferon Res.9:591-602.

53 Hockertz S., G. Franke, I. Paulini and M. L. Lohman-matthes. 1991. Immunotherapy of murine visceral leishmaniasis with murine recombinant interferon-gamma and MTP-PE encapsulated in liposomes. J. Ineterferon Res. 11:177-185.

54 Kierzenbaum F. and R. W. Ferraresi. 1979. Enancement of host resistance against *Trypanosoma cruzi* infection by the immunoregulatory agent muramyl dipeptide. Infect. Immun. 25:273-278.

55 Munoz-Fernandez M. A., M. A. Fernandez and M. Fresno. 1992. Activation of human macrophages for the killing of intracellular *Trypanosoma cruzi* by TNF-alpha trought a nitric oxide-dependent mechanism. Immunol. Lett. 33:35-40.

56 Reed S. G. 1988. In vivo administration of recombinant ineterferon gamma induces macrophage activation, and prevent acute disease, immune suppresion, and death in experimental *Trypanosoma cuzi* infections. J. Immunol. 140:4342-4347.

57 Badger A. M., J. S. Hutchman, C. P. Sung and P. J. Bugelski. 1987. Activation of alveolar macrophages by gamma interferon to inhibit *Toxoplasma gondii* in vitro. J. Leukoc. Biol. 42:447-454.

58 Mellors J. W., R. J. Debs and J. L Ryan. 1989. Incorporation of recombinant gamma interferon into liposomes enhances its ability to induce peritoneal macrophage antitoxoplasma activity. Infect. Immun. 57:132-137.

59 Seger, M., D. Gold, J. Lengy, H. Pauli, and Y. Keisari. 1992. On the interaction between

macrophages and development stages of *Schistosoma mansoni*: effect of muramyl tripeptide phosphatidyl ethanolamine (MTP-PE) treatment on mice survival and the generation of schistosomulicidal macrophages. Parasite Immunol. 14:355-369.

60 The International Chronic Granulomatous Disease Cooperative Study Group. 1991. A controlled trial of interferon gamma to prevent infection in chronic granulomatous disease. N. Engl. J Med. 324:509-516.

61 Baron, S., S.K. Tyring, W.R. Fleischmann et al. 1991. The interferons: mechanisms of action and clinical applications. JAMA 266:1375-1383.

62 Billiau, A., and R. Dijkmans. 1990. Interferon-γ: Mechanism of action and therapeutic potential. Biochem. Pharmacol. 40:1433-1439.

63 Czarniecki, C.W., and G. Sonnenfeld. 1993. Interferon-gamma and resistance to bacterial infections. APMIS. 101:1-17.

64 IJzermans, J.N.M., and R.L. Marquet. 1989. Interferon-gamma: A review. Immunobiol. 179:456-473.

65 Murray, H.W. 1988. Interferon-gamma, the activated macrophage, and host defence against microbial challenge. Ann. Intern. Med. 106:595-608.

66 Williams, J.G., G.J. Jurkovich, and R.V. Maier. 1993. Interferon-γ: A key immunoregulatory lymphokine. J. Surg. Res. 54:79-93.

67 Cheadle, W.G., M.J. Hershman, B. Mays, L. Melton, and H.C. Polk Jr. 1989. Enhancement of survival from murine polymicrobiol peritonitis with increased abdominal abscess formation. J. Surg. Res. 47:120-123.

68 Ding JW, R Andersson, B Hultberg, V Soltesz, S Bengmark. 1993. Modification of reticuloendothelial function by muramyl dipeptide-encapsulated liposomes in jaundiced rats treated with biliary decompression. Scand. J. Gastroenterol. 28:53-62.

69 Dunn, C.W., M.D. and J.W. Horton. 1990. Muramyl dipeptide improves mononuclear phagocyte system function in obstructive jaundice. J. Surg. Res. 48: 249-253.

70 Friedman, H., and G. Warren. 1984. Muramyl dipeptide-induced enhancement of phagocytosis of antibiotic pretreated *Escherichia coli* by macrophages. Proc. Soc. Exp. Biol. Med. 176:366-370.

71 Lamont, P.M., L.S. Trachtenberg, C.S. West, and H.C. Polk Jr. 1983. Nonspecific host defence stimulation and antibiotic-resistance infection. J. Antimicrob. Chemother. 12:S117-S122.

72 Matsumoto K., H. Ogawa, T. Kusama, et. al. 1981. Stimulation of nonspecific resistance to infection induced by 6-O-acyl muramyl dipeptide analogs in mice. Infect. Immun. 32, 748-758.

73 Matsumoto, K., H. Ogawa, O. Nagase, T. Kusama, and I. Azuma. 1981. Stimulation of nonspecific host resistance to infection induced by muramyldipeptides. Microbiol. Immunology. 25:1047-1058.

74 Nimmanwudipong, T., W.G. Cheadle, S.H. Appel, H.C. Polk Jr. 1992. Effect of protein malnutrition and immunomodulation on immune cell populations. J. Surg. Res. 52:233-238.

75 Stellato, T.A., M.C. Townsend, N. Gordon, L.H. Danziger, P. Galloway, N.L. Hawkins, and D.E. Fry. 1988. Effects of muramyl dipeptide and core body temperature on peritoneal bacterial clearance. Arch. Surg. 123:465-469.

76 Matsumoto, K., T. Otani, T. Une, Y. Osada, H. Ogawa, and I. Azuma. 1983. Stimulation of nonspecific resistance to infection induced by muramyl dipeptide analogs substituted in the gamma-carboxyl group and evaluation of N alpha-muramyl dipeptide-N epsilon stearoyllysine. Infect. Immun. 39:1029-1040.

77 Osada Y., M. Mitsuyama. T. Une, K. Matsumoto, T. Otani, M. Satoh, H. Ogawa and K. Nomoto. 1982. Effect of L18-MDP(Ala), a synthetic derivative of muramyl dipeptide, on nonspecific resistance of mice to microbial infections. Infect. Immun. 37:292-300.

78 Osada, Y., T. Otani, M. Sato, T. Une, K. Matsumoto, and H. Ogawa. 1982. Polymorphonuclear leukocyte activation by a synthetic muramyl dipeptide analog. Infect. Immun. 38:848-854.

79 Ding, J.W., R. Andersson, V.L. Soltesz, H. Parsson, K. Johansson, W. Wang, and S. Bengmark. 1994. Inhibition of bacterial translocation in obstructive jaundice by muramyl tripeptide phosphatidylethanolamine in the rat. J. Hepatol. 20:720-728.

80 Cheadle, W.G., K. Hanasawa, R.N. Gallinaro, T. Nimmanwudipong, M. Kodama, and H.C. Polk Jr. 1991. Endotoxin filtration and immune stimulation improve survival from Gram-negative sepsis. Surgery 110:785-791.

81 Cross, A., L. Asher, M. Seguin, et al. 1995. The importance of a lipopolysaccharide-initiated, cytokine mediated host defence mechanism in mice against extraintestinally invasive *Escherichia*

coli. J. Clin. Invest. 96:676-686.

82 Fu, Y.K., S. Arkins, Y.M. Li, R. Dantzer, and K.W. Kelley. 1994. Reduction in superoxide anion secretion and bacterial activity of neutrophils from aged rats: reversal by the combination of gamma interferon and growth hormone. Infect. Immun. 62:1-8.

83 Gennari, R., J.W. Alexander, and T. Eaves-Pyles. 1994. IFN-gamma decreases translocation and improves survival following transfusion and thermal injury. J. Surg. Res. 56:530-536.

84 Malangoni, M.A., D.H. Livingston, G. Sonnenfeld, and H.C. Polk Jr. 1990. Interferon gamma and tumor necrosis factor alpha. Use in gram-negative infection after shock. Arch. Surg. 125:444-446.

85 Ausobsky J. R., C. Leclerc and L. Chedid. 1984. Muramyl depeptide increases tolerance to shock and bacterial challenge in mice. Br. J. Surg. 71:151-153.

86 Ausobsky, J.R., M. Scuitto, L.S. Trachtenberg, and H.C. Polk Jr. 1984. The role of muramyl dipeptide in the therapy of established experimental bacterial infection. Br. J. Exp. Path. 65:1-9.

87 Chedid, L., M. Parant, F. Parant, P. Lefrancier, J. Choay, and E Lederer. 1977. Enhancement of Nonspecific Immunity to *Klebsiella pneumoniae* Infection by a Synthetic Immunoadjuvant (N-Acetylmuramyl-L-alanyl-D-isoglutamine) and Several Analogs. Proc. Natl. Acad. Sci. USA. 74:2089-2093.

88 Chedid, L., M.A. Parant, F.M. Audibert, et al. 1982. Biological activity of a new synthetic muramyl peptide adjuvant devoid of pyrogenicity. Infect. Immun. 35:417-424.

89 Cobb J. P., C. M. Brown, G. L. Brown and H. C. Polk Jr. 1986. Muramyl dipeptide protects decomplemented mice from surgicaly induced infection. Int. J. Immunopharmacol. 8:799-803.

90 Gaar, E., W. Naziri, W.G. Cheadle, J.D. Pietsch, M. Johnson, and H.C. Polk Jr. 1994. Improved survival in stimulated surgical infection with combined cytokine, antibiotic and immunostimulant therapy. Br. J. Surg. 81:1309-1311.

91 Galland R.B., L.S. Trachtenberg, N. Rynerson, and H.C. Polk Jr. 1983. Nonspecific enhancement of resistance to local bacterial infection in starved mice. Arch. Surg. 118:161-164.

92 Galland, R.B., K.J. Heine, and H.C. Polk. 1983. Nonspecific stimulation of host defenses against bacterial challenge in immunosuppressed mice. Arch. Surg. 118:333-337.

93 Galland, R.B., and H.C. Polk. 1982. Non-specific stimulation of host defenses against a bacterial challenge in malnourished hosts. Br. J. Surg. 69:665-668.

94 Parant M., F. Parant and L. Chedid. 1978. Enhancement of the neonate's nonspecific immunity to Klebsiella infection by muramyl dipeptide, a synthetic immunoadjuvant. Proc. Natl. Acad. Sci. USA 75:3395-3399.

95 Parant M. and L. Chedid. 1985. Stimulation of non-specific resistance to infections by synthetic immunoregulatory agents. Infection 13:S251-255.

96 Polk Jr H. C., J. H. Calhoun, M. Eng, et. al. 1981. Nonspecific enhancement of host defences against infection: Experimental evidence of a new order of efficacy and safety. Surgery 90:376-380.

97 Polk Jr HC, RB Galland, and FR Ausobsky. 1982. Nonspecific enhancement of resistance to bacterial infection. Evidence of an effect supplemental to antibiotics. Ann. Surg. 196:436-441.

98 Polk Jr H. C., P. M. Lamont and R. B. Galland. 1990. Containment as a mechanism of non-specific enhancement of defenses against bacterial infection. Infect. Immun. 58:1807-1811.

99 Tatara, O., C. Nakahama, and Y. Niki. 1992. Synergistic effects of romurtide and cefmenoxime against experimental *Klebsiella pneumoniae* in mice. Antimicrob. Agents Chemother. 36:167-171.

100 Yapo, A., J.F. Patit. E. Lederer, M. Parant, F. Parant, and L. Chedid. 1982. Fate of two 14C labelled muramyl peptides: Ac-Mur-L-Ala-gamma-D-Glu-meso-A2pm and Ac-Mur-L-Ala-gamma-D-Glu-meso-A2pm-D-Ala-D-Ala in mice. Evaluation of their ability to increase non specific resistance to Klebsiella infection. Int. J. Immunopharmacol. 4:143-149.

101 Brewer, R.J., R.B. Galland, and H.C. Polk Jr. 1982. Amelioration by muramyl dipeptide of the effect of induced hyperferremia upon Klebsiella infection in mice. Infect. Immun. 38:175-178.

102 Melissen, P.M.B., W. van Vianen, Y. Rijsbergen, and I.A.J.M. Bakker-Woudenberg. 1991. Free versus liposome-encapsulated muramyl tripeptide phosphatidylethanolamine in treatment of experimental *Klebsiella pneumoniae* infection. Infect. Immun. 60:95-101.

103 Melissen, P.M.B., W. van Vianen, and I.A.J.M. Bakker-Woudenberg. 1992. Roles of peripheral leukocytes and tissue macrophages in antibacterial resistance induced by free or liposome-encapsulated muramyl tripeptide phosphatidylethanolamide. Infect. Immun. 60:4891-4897.

104 Melissen, P.M.B., W. van Vianen, and I.A.J.M. Bakker-Woudenberg. 1994. Treatment of *Klebsiella*

pneumoniae septicemia in normal and leukopenic mice by liposome-encapsulated muramyl tripeptide phosphatidylethanolamide. Antimicrob. Agents. Chemother. 38:147-150.

105 Hagen ten TLM, W van Vianen, and IAJM Bakker-Woudenberg. 1995. Modulation of nonspecific antimicrobial resistance of mice to *Klebisella pneumoniae* septicemia by liposome-encapsulated muramyl tripeptide phosphatidylethanolamine and interferon–γ alone or combined. J. Infect. Dis. 171:385-392.

106 Hershman, M.J., H.C. Polk, J.D. Pietsch, E. Shields, S.R. Wellhausen, and G. Sonnenfeld. 1988. Modulation of infection by gamma interferon treatment following trauma. Infect. Immun. 56:2412-2416.

107 Hershman, M.J., H.C. Polk, J.D. Pietsch, D. Kuftinec, and G. Sonnenfeld. 1988. Modulation of *Klebsiella pneumoniae* infection of mice by interferon-γ. Clin. Exp. Immunol. 72:406-409.

108 Hershman, M.J., G. Sonnenfeld, B.W. Mays, F. Flemming, L.S. Trachtenberg, and H.C. Polk. 1988. Effects of interferon-γ treatment on surgically stimulated wound infection in mice. Microbial Pathogen. 4:165-168.

109 Hershman, M.J., G. Sonnenfeld, W.A. Logan, J.D. Pietsch, S.R. Wellhausen, and H.C. Polk Jr. 1988. Effect of interferon-gamma treatment on the course of a burn wound infection. J. Interferon Res. 8:367-373.

110 Fil'chakov, I.V. L.V. Avdeeva, F.V. Fil'chakov, N.I. Spivak, E.I. Protsap, and V.K. Ivanenko. 1992. Antibacterial effects of gamma-interferon in experimental Klebsiella infection. Antibiot. Khimiother. 37:31-34.

111 Chedid L., M. Parant, F. Parant, et al. 1979. Enhancement of certain biological activities of muramyl dipeptide derivatives after conjugation to a multi-poly(DL-alanine)-poly(L-lysine) carrier. Proc. Natl. Acad. Sci. USA 76:6557-6561.

112 Furuya, T., Y. Kumazawa, H. Takimoto, et al. 1989. Immunostimulatory activity of 1-O-acylated muramyl dipeptides, with or without a 6-O-phosphoryl group, in aqueous form. Int. J. Immunopharmacol. 11:35-43.

113 Parant M. A., F. M. Audibert, L. Chedid, et. al. 1980. Immunostimulant activities of a lipophilic muramyl dipeptide derivative and of desmuryl peptidolipid analogs. Infect. Immun. 27:826-831.

114 Stinnett, J.D. L.D. Loose, P. Miskell, C.L. Tenney, S.J. Gonce, and J.W. Alexander. 1983. Synthetic immunomodulators for prevention of fatal infections in a burned guinea pig model. Ann. Surg. 198:53-57.

115 Fraser-Smith, E.B., and T.R. Metthews. 1981. Protective effect of muramyl dipeptide analogs against infections of Pseudomonas aeruginosa or Candida albicans in mice. Infect. Immun. 34:676-683.

116 Otani, T. K. Katami, T. Une, Y. Osada, and H. Ogawa. 1984. Restoration by MDP-Lys (L18) of resistance to Pseudomonas pneumonia in immunosuppressed guinea pigs. Microbiol. Immunology. 28:1077-1082.

117 Ozaki, T., M. Maeda, H. Hayashi, Y. Nakamura, H. Moriguchi, T. Kamei, S. Yasuoka, and T. Ogura. 1989. Role of alveolar macrophages in the neutrophil-dependent defense system against *Pseudomonas aeruginosa* infection in the lower respiratory tract. Amplifying effect of muramyl dipeptide analog. Am. Rev. Respir. Dis. 140:1595-1601.

118 Pierangeli, S.S., H.C. Polk Jr, M.J. Parmely, and G. Sonnenfeld. 1993. Murine interferon-gamma enhances resistance to infection with starins of *Pseudomonas aeruginosa* in mice. Cytokine 5:230-234.

119 Speert, D.P. and L. Thorson. 1991. Suppression by human recombinant gamma interferon of in vitro macrophage nonopsonic and opsonic phagocytosis and killing. Infect. Immun. 59:1893-1898.

120 Phillips, N.C., and L. Chedid. 1987. Anti-infectious activity of liposomal muramyl dipeptides in immunodeficient CBA/N mice. Infect. Immun. 55:1426-30.

121 Onozuka K., T. Saito and M. Nakano. 1984. Augmentation of protective and antibacterial activity induced by muramyl dipeptides in CBA/N defective mice with X-linked immunodeficiency for *Salmonella enteritidis* infection. Infect. Immun. 45:424-427.

122 Onozuka, K., T. Saito, and M. Nakano. 1984. Effect of muramyl dipeptide analog on *Salmonella enteritidis* infection in beige mice with Chediak-Higashi syndrome. Microbiol. Immunology. 28:1211-1221.

123 Edwards III, C.K., S.M. Ghiasuddin, L.M. Yunger, R.M. Lorence, S. Arkins, R. Dantzer, and K.W. Kelley. 1992. In vivo administration of recombinant growth hormone or gamma interferon activates

Macrophages: Enhanced resistance to experimental *Salmonella typhimurium* infection is correlated with generation of reactive oxygen intermediates. Infect. Immun. 60:2514-2521.

124 Izadkhah, Z., A.D. Mandel, and G. Sonnenfeld. 1980. Effect of treatment of mice with sera containing gamma interferon on the course of infection with *Salmonella typhimurium* strain LT-2. J. Interferon Res. 1:137-145.

125 Matsumura H., K. Onozuka, Y. Terada, Y. Nakano and M. Nakano. 1990. Effect of murine recombinant interferon-gamma in the protection of mice against Salmonella. Int. J. Immunopharmacol. 12:49-56.

126 Sasahara, T., N. Ikewaki, H. Tamauchi, and N. Osawa. 1992. Oxygen-independent antimicrobial activity against *Salmonella enteritidis* of specially activated macrophages with living vaccine. Kitasato Arch. Exp. Med. 65:225-237.

127 Bukholm, G., B.P. Berdal, C. Haug, and M. Degre. 1984. Mouse fibroblast interferon modifies *Salmonella typhimurium* infection in infant mice. Infect. Immun. 45:62-66.

128 Fil'chakov, I.V., F.V. Fil'chalov, N.I. Spivak, E.I. Protsap, and V.K. Ivanenko. 1994. Gamma-interferon and the antibacterial activity of mouse macrophages in a Salmonella infection. Zh. Mikrobiol. Epidemiol. Immunobiol. Jan-Feb:64-69.

129 Ishibashi, Y. And T. Arai. 1990. Effect of gamma-interferon on phagosome-lysosome fusion in *Salmonella typhimurium*-infected murine macrophages. FEMS Microbiol. Immunol. 2:75-82.

130 Kagaya R.L., K. Watanabe and Y. Fukazawa. 1989. Capacity of recombinant gamma interferon to activate macrophages for Salmonella-killing activity. Infect. Immun. 57:609-615.

131 Langermans, J.A., P.H. Nibbering M.E. van der Hulst, and R. Van Furth. 1991. Microbicidal activities of *Salmonella typhimurium*- and interferon-gamma-activated mouse peritoneal macrophages. Pathobiology 59:189-193.

132 Muotiala, A., and P.H. Makela. 1990. The role of IFN-gamma in murine *Salmonella typhimurium* infection. Microb. Pathog. 8:135-141.

133 Osanto, S., M.T. van den Barselaar, and J.T. van Dissel. 1991. Increased intracellular killing of bacteria in vitro by monocytes of patients with metastatic melanoma before and during treatment with interferon-gamma and interferon-alpha. Eur. J. Cancer. 27:478-482.

134 Spivak, N.I., I.V. Fil'chakov, E.V. Bratus, L.A. Ganova, and V.S. Zueva. 1988. Changes in the bactericidal activity of macrophages from mice with various degrees of resistance to *Salmonella typhimurium* infection as affected by interferon. Zh. Mikrobiol. Epidemiol. Immunobiol. May:81-84.

135 Pierangeli SS, G Sonnenfeld. 1993. Treatment of murine macrophages with murine interferon-gamma and tumour necrosis factor-alpha enhances uptake and intracellular killing of *Pseudomonas aeruginosa*. Clin. Exp. Immunol. 93:165-171.

136 Quiroga, G.H., W.E. Owens, and S.C. Nickerson. 1992. Response of Heifer mammary gland macrophages and neutrophils to interferon-gamma stimulation in vitro. Can. J. Vet. Res. 57:212-214.

137 Parant, M., A. Galelli, F. Parant, and L. Chedid. 1976. Role of B-lymphocytes in nonspecific resistance to *Klebsiella pneumoniae* infection of endotoxin-treated mice. J. Infect. Dis. 134:531-539.

138 Hershman, M.J., J.D. Pietsch, L. Trachtenberg, T.H.R. Mooney, R.E. Shields, and G. Sonnenfeld. 1989. Protective effects of recombinant human tumour necrosis factor a and interferon γ against surgically simulated wound infection in mice. Br. J. Surg. 76:1282-1286.

139 Nauciel, C. , and F. Espinasse-Maes. 1992. Role of gamma interferon and tumor necrosis factor alpha in resitance to *Salmonella typhimurium* infection. Infect. Immun. 60:450-454.

140 Silva, A.T., and J. Cohen. 1992. Role of interferon-γ in experimental Gram-negative sepsis. J. Infect. Dis. 166:331-335.

141 Pak, C.C., and I.J. Fidler. 1991. Liposomal delivery of biological response modifiers to macrophages. Biotherapy 3:55-64.

142 Melissen, P.M.B., W. van Vianen, P.J.M. Leenen, and I.A.J.M. Bakker-Woudenberg. 1994. Tissue distribution and cellular distribution of liposomes encapsulating muramyltripeptide phosphatidyl ethanolamide. Biotherapy 7: 71-78.

143 Schumann, G., P. van Hoogevest, P. Frankhauser et al. 1989. Comparison of free and liposomal MTPPE: pharmacological, toxicological and pharmacokinetic aspects. In: Lopez-Berestein, G., I.J. Fidler eds. Liposomes in the Therapy of Infectious Disease and Cancer. UCLA, Alan R. Liss, Inc. New York, Symposium on Molecular and Cellular Biology, New Series, 89:191-203.

144 Phillips, N.C., and L. Chedid. 1988. Muramyl peptides and liposomes. In: G. Gregoriadis ed.

Liposomes as Drug Carriers.Wiley & Sons Ltd. p. 243-259.

145 Gay, B., J-M. Carbot, C. Schnell. P. van Hoogevest, and D. Gygax. 1993. Comparative pharmacokinetics of free muramyl tripeptide phosphatidyl ethanolamine (MTP-PE) and liposomal MTP-PE. J. Pharm. Sci. 82:997-1001.

146 Raz, A., C. Bucana, W.E. Fogler, G. Poste, and I.J. Fidler. 1981. Biochemical, morphological, and ultrastructural studies on the uptake of liposomes by murine macrophages. Cancer Res. 41:487-494.

147 Schumann, G.P., P. van Hoogevest, P. Fankhauser, et al. 1989. Comparison of free and liposomal mtppe: pharmacological, toxicological and pharmacokinetic aspects. In: Lopez-Berestein, G., I.J. Fidler eds. Liposomes in the Therapy of Infectious Disease and Cancer. UCLA, Alan R. Liss. Inc. New York, Symposium on Molecular and Cellular Biology, New Series, 89:191-203.

148 Melissen, P.M.B., W. van Vianen, O. Bidjai, M. van Marion, and I.A.J.M. Bakker-Woudenberg. 1993. Free versus liposome-encapsulated muramyl tripeptide phosphatidylethanolamide (MTPPE) and interferon-γ (IFN-γ) in experimental infetion with *Listeria monocytogenes*. Biotherapy 6:113-124.

149 Bennett, C.L., N.J. Vogelzang, M.J. Ratain, and S.D. Reich. 1986. Hyponatremia and other toxic effects during a phase I trial of recombinant human gamma interferon and vinblastine. Cancer Treat. Rep. 70:1081-1084.

150 Gutterman, J.U., M.G. Rosenblum, A. Rios, H.A. Fritsche, and J.R. Quesada. 1984. Pharmacokinetic study of partially pure gamma-interferon in cancer patients. Cancer Res. 44:4164-4171.

151 Kurzrock, R., M.G. Rosenblum, S.A. Sherwin, A. Rios, M. Talpaz, J.R. Quesada, and J.U. Gutterman. 1985. Pharmacokinetics, single-dose tolerance, and biological activity of recombinant γ-interferon in cancer patients. Oncology 42:41-50.

152 Frasersmith, E.B., R.V. Waters, and T.R. Matthews. 1982. Correlation between in vivo anti-Pseudomonas and anti-Candida activities and clearance of carbon by the reticuloendothelial system for various muramyl dipeptide analogs, using normal and immunosuppressed mice. Infect. Immun. 35:105-110.

153 Izbicki JR, C Readler, A Anke et al. 1991. Beneficial effect of lipsome-encapsulated muramyl-tripeptide in experimental septicemia in a porcine model. Infect Immun. 59:126-30.

154 Fidler, I.J., W.E. Fogler, E.S. Kleinerman, and I. Saiki. 1985. Abrogation of species specificity for activation of tumoricidal properties in macrophages by recombinant mouse or human interferon-γ encapsulated in liposomes. J. Immunol. 135:4289-4296.

155 Mehta, K., R.L. Juliano, and G. Lopez-Berestein. 1984. Stimulation of macrophage protease secretion via liposomal delivery of muramyl dipeptide derivatives to intracellular sites. Immunology 51:517-527.

156 Smith, M.R., K. Muegge, J.R. Keller, H-F. Kung, H.A. Young, and S.K. Durum. 1990. Direct evidence for an intracellular role for IFN–γ: microinjection of human IFN–γ induces Ia expression on murine macrophages. J. Immunol. 144:1777-1782.

157 McGowan JE, PJ Chesney, KB Crossley, and FM LaForce. 1992. Guidelines for the use of systemic glucocorticosteroids in the management of selected infections. J. Infect. Dis. 165:1-13.

158 Turk JL, and D Parker. 1982. Effect of cyclophosphamide on immunological control mechanisms. Immunol. Rev. 65:99-113.

159 Langermans, J.A.M., van der Hulst, M.E.B., Nibbering, P.H., Hiemstra, P.S., Fransen, L., and van Furth, R. 1992. IFN-γ-induced L-arginine dependent toxoplasmastatic activity in murine peritoneal macrophages is mediated by endogenous tumor necrosis factor–α. J. Immunol. 48:568-574.

160 Neta, R., Sayer, T.J., and Oppenheim, J.J. 1992. Relationship of TNF to interleukins. Immunology Series 56:499-566.

161 O'Reilly, T., and Zak, O. 1991. Enhancement of the effectiveness of antimicrobial therapy by muramyl pepetide immunomodulators. Clin. Infect. Dis. 14:1100-1109.

162 Murray HW, J Hariprashad, B Aguero, T Arakawa, and H Yeganegi. 1995. Antimicrobial response of a T cell-deficient host to cytokine therapy: Effect of interferon–γ in experimental visceral Leishmaniasis in nude mice. J. Infect. Dis. 171:1309-1316.

163 Kleinerman, E.S., J.L. Murray, J.S. Snyder, J.E. Cunningham, and I.J. Fidler. 1989. Activation of tumoricidal properties in monocytes from cancer patients following intravenous administration of liposomes containing muramyl tripeptide phophatidylethanolamine. Cancer Res. 49:4665-4670.

SUMMARY

Severe infections represent a continuing threat to immunocompromised patients. The availability of a broad spectrum of antibiotics and the large number of studies on optimal dosing and administration protocols have not taken this threat away. Among patients who develop complications of shock and organ failure, mortality can reach 90%. Septicemia thus represents a leading cause of death in the developed countries, and its incidence has increased significantly over the past decade. Especially in patients with a suppressed host defense, antimicrobial therapy of septicemic infection often fails. Cure rates from infection is poorest among those patients who have persistent neutropenia. Various strategies are available to improve treatment of immunodeficient patients with severe infections. One strategy is to intensify antibiotic treatment by applying more drugs at the same time; this is however not always effective. Failure of host defense to support antibiotic treatment allows this to happen. Another possible way to improve therapeutic results might thus be to stimulate host defense, either nonspecific defense (granulocytes and macrophages) or specific humoral and cellular defense. Activation of nonspecific host defense has the advantages that it can be effective in different types of infection, and does not induce tolerance of the microorganisms to treatment. Activation of nonspecific host defense can be achieved with biologic or synthetic immunomodulators that influence or modify (parts of) the innate resistance in a direct or indirect way, independently of challenge. Many different agents have been tested for their immunomodulatory capacity.

The aim of the present study was the development of a treatment which could improve outcome of patients with a serious infection, by increasing host defense. Because, impressive up regulation of host defense could be achieved, the mechanisms studied which underlie host defense potentiation by immunomodulators. The goal was to learn more about immunomodulation, giving us tools to improve antimicrobial host defense potentiation even better.

As stated above, agents which have the capacity to stimulate host defense are called immunomodulators. One might prefer immunostimulators, but as will become clear from the present study the applied agents: muramyl tripeptide phosphatidylethanolamine (MTPPE) and interferon-gamma (IFN–γ) also strongly influence the type of immune response which is boosted by these agents. We used MTPPE and IFN–γ in a liposome-encapsulated form. Liposomal encapsulation has several advantages: prolonged half-life time of agents in the body, high concentrations at specific sites can be reached, toxicity can be reduced and by co-encapsulation of agents synergy in vivo can be assured. As macrophages are believed to be the most important target cells for immunomodulation the use of classical liposomes, which tend to localize in large numbers in these cells, is obvious. In this thesis mice were treated with liposome-encapsulated immunomodulators and their resistance to *Klebsiella pneumoniae* infections was studied. The immunologic response which follows the treatment was investigated in more detail.

In chapter two we show that treatment of mice with liposome-encapsulated MTPPE (LE-MTPPE) or IFN–γ (LE-IFN–γ) improved host resistance dramatically to a *K. pneumoniae* septicemia. Moreover, combination of agents encapsulated in liposomes (LE-MTPPE/IFN–γ) resulted in 100% survival of mice from a normally lethal septicemia. Strikingly, stimulation of macrophages in vitro did not result in increased killing of intracellular bacteria,

although macrophages are thought to be the primary target for treatment. These results indicate that immunomodulation is very potent, however increased host defense is not solely due to activation of macrophages.

Therefore, involvement of other cells in immunomodulation was investigated (chapter three). It appeared that T cells play a key role in activation of host defense with immunomodulators. Moreover, endogenous IFN–γ production appeared essential for immunomodulators to be effective. From this we concluded that Th1, NK and $CD8^+$ cells are involved in immunomodulation with our agents. Ex vivo studies revealed that indeed immunomodulation manipulated the immune response towards a Th1 cell response.

Knowing the involvement of T cells in potentiation of host defense by immunomodulators, did not however provide us with information on why increased host defense is obtained without increased potency of macrophages to take up or kill intracellular bacteria. In chapter four the effect of immunomodulators on immunologically important cell populations of liver, spleen, blood and bone marrow was studied. Administration of LE-MTPPE/IFN–γ resulted in a strongly increased hemopoietic cell numbers in liver and spleen. Especially the myeloid cell numbers (granulocytes and macrophages) were augmented. Most strikingly also erythropoiesis was strongly increased in spleen. An increased hemopoiesis in bone marrow was observed upon immunomodulation, which was probably responsible for migration of cell to the periphery. In contrast to spleen, administration of immunomodulators resulted in a decline in erythropoiesis in bone marrow. Based on these resulted we hypothesized that an increase in effector cells (quantity), rather than stimulation of the single effector cell (quality), is responsible for increased host defense upon immunomodulation.

Taken together, the results presented in chapter three and four indicated that several cell populations are involved in immunomodulation, and that a massive immunologic process is induced by liposome-encapsulated MTPPE and IFN–γ. Important therefore would be to understand which cytokines play a role in this process and at what time point these cytokines are produced. In chapter five a first step was made to study the expression of important immunoregulatory cytokines after immunomodulation. Mice were treated with LE-MTPPE/IFN–γ after which mRNA expression levels were determined in liver and spleen by reverse transcriptase PCR. Administration of immunomodulators resulted in increased IL-10 mRNA expression in spleen, after an initial decline, at 96 h after end of treatment. Strikingly we were unable to demonstrate expression of TNF–α or IL-6 mRNA, two cytokine known to be important in inflammatory processes and produced by macrophages. We speculate that the time point of measurement, after five injections of immunomodulator, was too late. This would indicate that activation of macrophages by immunomodulators is transient (within 12 h after injection) or takes place only after the first rounds of injections with immunomodulator. IL-2 mRNA expression indicated that indeed T cells are involved in immunomodulation as already shown in chapter three.

Effective stimulation of antimicrobial host defense with immunomodulators seems very important in immunodeficient patients which are prone to severe infections. A likely group of patients which might benefit from this treatment are transplant recipients. These patients are rendered immunodeficient on purpose in order to prevent rejection of transplants. It is known at what moment these patients become immunodeficient, which is highly relevant because immunomodulators are most effective when given prophylactically. Moreover, immunomodulation is less active when administered in already immunodeficient patients. However, a major risk factor in this patient group might

be that activation of antimicrobial host defense might have a negative effect on transplants. Meaning, administration of immunomodulators in order to help the patient fight infections might cause rejection of the graft and therefore seriously threaten patients life. In chapter six we studied the effect of immunomodulation on skin graft rejection in mice. It was found that host defense activation with LE-MTPPE, LE-IFN–γ or even LE-MTPPE/IFN–γ did not affect skin graft rejection. Both in immunocompetent and in T cell depleted mice effective enhancement of the host resistance could not accelerate skin graft rejection. From this study we conclude that potent immunomodulation is possible in graft-bearing hosts without any negative effect on the graft.

The positive results obtained on effective antimicrobial host defense activation as presented in chapter two, and the indication that macrophages are involved but can not explain for the results led us to investigate the role of macrophages in immunomodulation in more detail. Although macrophages were shown not to respond with increased uptake and killing of bacteria in vitro, in vivo results demonstrated that depletion of macrophages before immunomodulation is detrimental for treatment. The in vitro studies as described in chapter two were performed with peritoneal macrophages. We speculate that macrophages which are mainly involved in immunomodulation are located in liver and spleen. Therefore attempts were made to isolate macrophages from spleen and liver to investigate the antimicrobial behavior of these cells in vitro. In chapter seven isolation of liver macrophages (Kupffer cells, KC) and spleen macrophages is presented. Using counterflow centrifugation elutriation (CCE) we were able to isolated large numbers of KC in high purity. Also an increased purification of spleen macrophages was achieved by using this technique. Macrophages were isolated with high viability and could be cultured for several weeks. Determination of purification with specific monoclonal antibodies, enzymatically or histologically demonstrate high correlation between tests. It was also demonstrated that macrophage number in populations could easily be determined by autofluorescence on a flowcytometer. Using CCE we were able to separate KC and splenic macrophages on size into three fractions. Functionality assays in vitro demonstrated that macrophages in different fractions exhibited different activities related to their size. Especially small KC displayed increased oxygen metabolism upon exposure to heat killed bacteria. This was also seen for splenic macrophages. Nitric oxide (NO) and TNF–α on the other hand where mainly produced by larger KC upon exposure to heat killed bacteria. All KC fractions however exhibited comparable phagocytic activity. These results indicate that macrophages do not only differ in activity between types, but that there is also differences between macrophage activity within a population. This was found to correlate with findings of others demonstrating that KC are located at different sites in liver, which strongly correlates with their size.

In chapter eight heterogeneity of KC was studied in relation to immunomodulation. Three, by CCE isolated KC subfractions (small, intermediate, and large) were studied on their capacity to produce NO and TNF–α upon priming with IFN–γ and/or TNF–α and subsequent exposure to heat killed bacteria. As stated before small and intermediate KC produce only little TNF–α or NO. Large KC on the other hand produce significant amounts of NO and TNF–α after exposure to heat killed bacteria. Priming of KC before exposure to bacteria with IFN–γ increased TNF–α production in large KC and NO production in all groups. Priming of KC with TNF–α dramatically increased TNF–α production of small KC, but had only little effect on NO production. Priming of KC with combination of TNF–α

and IFN–γ before exposure to bacteria strikingly resulted in reduced TNF–α production as compared with TNF–α primed cells. NO production was however enhanced in all groups in a synergistic way by the combination of TNF–α and IFN–γ. These results demonstrate extensive heterogeneity among KC with respect to immunologic response to stimuli. Clearly KC have different functions in immunologic reactions which seem to be related to size.

Immunomodulation to lethal bacterial septicemia can be very effective, and as shown in the experimental infection model used in the present thesis, resulting in 100% survival. In this process macrophages seem to play a key role, but also other cells (e.g. T cells) are important. Extensive studies demonstrate that immunomodulation goes beyond macrophage activation and includes many other cell populations. The understanding of the mechanism behind immunomodulation is further complicated by the heterogeneity which already exists among KC as shown in this thesis. Further studies are necessary to complete the insight in the mechanisms behind host defense activation induced by immunomodulators. Hopefully this knowledge will lead to possibilities to combat severe infections especially in immunocompromised patients, where antibiotics are unable to provide adequate therapy.

SAMENVATTING

Ernstige infecties zijn nog steeds de oorzaak van hoge mortaliteit bij patiënten met een verminderde weerstand. De beschikking over een groot scala aan antibiotica en een veelheid aan studies naar optimale dosering hebben deze dreiging nog niet kunnen wegnemen. Onder patiënten die een septische shock ontwikkelen en daarbij behorend orgaan falen kan de mortaliteit oplopen tot 90%. Septische shock is daarom een belangrijke oorzaak van overlijden in de westerse wereld en neemt significant toe in de laatste tien jaar. Vooral in patiënten met een verminderde weerstand faalt de antimicrobiele behandeling van sepsis regelmatig. De kans op effectieve behandeling is vooral laag onder patiënten met een persisterende neutropenie. Voor deze patiënten zijn verschillende behandelingen voor handen. Een mogelijkheid is het intensifiëren van de antibiotica behandeling door meerdere middelen tegelijk toe te dienen, hetgeen echter niet altijd het gewenste resultaat geeft. Vooral falen van het eigen afweersysteem blijkt hierbij een belangrijke rol te spelen. Een andere manier om de therapie te doen slagen is daarom, het versterken van die eigen afweer: enerzijds de niet specifieke afweer (granulocyten en macrofagen) en anderzijds de humorale en specifiek cellulaire afweer. Activatie van de niet specifieke afweer heeft als voordeel dat het effectief kan zijn tegen meerdere verschillende infecties, en geen tolerantie induceert bij het micro-organisme tegen de behandeling. Activatie van de niet specifieke afweer kan verkregen worden met biologische of synthetische immunomodulatoren die instaat zijn om de eigen afweer te beïnvloeden (stimuleren, modificeren) direct of indirect, onafhankelijk van de infectie. Vele verschillende stoffen werden al reeds onderzocht op hun immunomodulerende capaciteit.

Het doel van de hier gepresenteerde studie is de ontwikkeling van een behandeling die de kans op herstel van een patiënt met een ernstige infectie moet vergroten door versterking van de eigen afweer. Vanwege de indrukwekkende resultaten die hiermee werden verkregen werden tevens de mechanismen bestudeerd die hieraan ten grondslag liggen. Het doel was om met meer inzicht nog betere behandelingen te ontwikkelen voor activatie van de eigen afweer tegen microbiële infecties.

Zoals reeds hierboven gezegd , worden stoffen die instaat zijn de eigen afweer te stimuleren immunomodulatoren genoemd. Men zou graag spreken van immunostimulatoren maar zoals duidelijk zal worden in de hier beschreven studies kunnen muramyl tripeptide phosphatidylethanolamine (MTPPE) en interferon-gamma (IFN–γ) ook sterke invloed hebben op het type immuunresponse die door deze stoffen wordt geactiveerd. Wij maakten gebruik van MTPPE en IFN–γ in een liposomaal ingekapselde vorm. Liposomale inkapseling heeft verschillende voordelen: verlenging van de halfwaardetijd in het lichaam, mogelijkheid tot hoge concentraties op specifieke plaatsen, reductie van de toxiciteit en mogelijkheid tot synergie in vivo door gelijktijdig inkapselen van meerdere stoffen in liposomen (co-inkapseling). Aangezien macrofagen worden verondersteld de belangrijkste doelcellen te zijn voor immunomodulatie, is het gebruik van klassieke liposomen, die vooral door macrofagen worden opgenomen, voor de hand liggend. In dit proefschrift werd de afweer van muizen tegen een *Klebsiella pneumoniae* infectie bestudeerd na behandeling met liposomaal ingekapselde immunomodulatoren. De immunologische reactie die na behandeling volgt werd tevens in meer detail onderzocht.

In hoofdstuk twee laten we zien dat behandeling van muizen met liposomaal ingekapseld MTPPE (LE-MTPPE) of IFN–γ (LE-IFN–γ) de afweer tegen een ernstige *K. pneumoniae* infectie effectief kan versterken. Bovendien resulteerde toepassing van deze stoffen tesamen in liposomen ingekapseld in 100% overleving van muizen van een normaliter letale infectie. Opvallend was dat stimulatie van macrofagen in vitro met deze stoffen niet resulteerde in een toegenomen opname of doding van bacteriën, hoewel macrofagen gezien worden als de belangrijkste cellen in dit proces. Deze resultaten tonen aan dat immunomodulatie zeer veel potentie heeft, maar dat de toename in de afweer van de gastheer niet te verklaren is door een toename in antimicrobiële activiteiten van de macrofagen.

Daarom werd een poging ondernomen om de betrokkenheid van andere cellen in het proces van immunomodulatie te onderzoeken (hoofdstuk drie). Het bleek dat naast macrofagen ook T cellen een belangrijke rol spelen in afweeractivatie door immunomodulatoren. Daarnaast bleek dat vooral endogeen IFN–γ van essentieel belang was voor effectieve immunomodulatie. Wij concludeerden hieruit dat met name Th1, NK en $CD8^+$ cellen een rol spelen in dit proces. Ex vivo experimenten bevestigde dit vermoeden en lieten zien dat immunomodulatie resulteert in verschuiving naar een door Th1 cellen gedomineerde immuun respons.

Alhoewel de betrokkenheid van de T cel nu aangetoond is, is er nog steeds onvoldoende informatie voor handen om te verklaren waarom een verhoogde afweer niet gepaard gaat met een toegenomen antimicrobiële activiteit van macrofagen. In hoofdstuk vier werd het effect van immunomodulatoren op belangrijke celpopulaties in de lever, milt, bloed en beenmerg bestudeerd. Toediening van LE-MTPPE/IFN–γ resulteerde in een sterke toename van hemopoietische cellen in de lever en milt. Vooral de myeloide cellen (granulocyten en macrofagen) waren toegenomen. Opvallend was de toenamen in erythroide cellen in de milt. Na immunomodulatie werd een toenamen in de hemopoiese waargenomen, die zeer waarschijnlijk verantwoordelijk is voor de migratie van cellen naar de periferie. In tegenstelling tot waarnemingen in de milt, resulteerde toediening van de immunomodulatoren in een afnamen van de erythropoiese in het beenmerg. Naar aanleiding van deze resultaten hebben wij de hypothese gesteld dat een toename van het aantal effector cellen (kwantiteit) van groot belangrijk is, terwijl een toename van de activiteit van de enkele effector cel (kwaliteit) slechts van ondergeschikt belang is.

Uit de resultaten in hoofdstuk drie en vier concluderen wij dat verscheidene celpopulaties betrokken zijn in het proces van immunomodulatie, en dat een massale immunologische reactie opgang is gebracht door liposomaal ingekapseld MTPPE and IFN–γ. Het is daarom belangrijk om meer inzicht te krijgen in de rol die bepaalde cytokinen spelen in dit proces en in welke sequentie deze cytokinen worden geproduceerd. In hoofdstuk vijf worden de eerste stappen beschreven die gemaakt werden voor de bestudering van de expressie van belangrijke immunoregulatoire cytokinen na immunomodulatie. Na behandeling van muizen met LE-MTPPE/IFN–γ werd de expressie van mRNA in lever en milt bepaald door middel van reverse transcriptase PCR. Toediening van immunomodulatoren resulteerde in een verhoging van de IL-10 mRNA expressie in de milt, na een initiële verlaging, op 96 uur na de laatste behandeling. Opvallend was de onmogelijkheid om expressie van TNF–α of IL-6 mRNA, twee cytokinen die belangrijk worden geacht in ontstekingsprocessen en vooral geproduceerd worden door macrofagen, aan te tonen. Wij denken dat de meetpunten, na vijfvoudige injectie van de immunomodulatoren, te laat zijn gekozen. Hieruit zouden we kunnen afleiden dat het proces van

macrofaag activatie door de immunomodulatoren transient is (binnen 12 uur na einde van behandeling) of dat wel meetbare macrofaag activatie plaats vindt alleen gedurende de eerste injecties met immunomodulator. De expressie van IL-2 mRNA duidt in ieder geval op de betrokkenheid van T cellen in het proces van immunomodulatie zoals al reeds in hoofdstuk drie werd aangetoond.

Effectieve stimulatie van de eigen afweer lijkt vooral belangrijk in patiënten met een verminderde algehele weerstand. Een groep patiënten die daarom vooral in aanmerking komt voor behandeling met immunomodulatoren zijn transplantatie patiënten. Deze patiënten krijgen stoffen toegediend die de eigen afweer verlagen om te voorkomen dat het lichaam het transplantaat afstoot. Het moment waarop deze patiënten immunodeficient worden is daarom bekend, wat van belang is aangezien immunomodulatie vooral effectief is als het profylactisch (voordat infecties optreden) wordt toegediend. Daarnaast zijn immunomodulatoren ook minder instaat de eigen afweer te stimuleren als deze bijvoorbaat al verzwakt is. Alhoewel daarom toediening van immunomodulatoren in deze patiënten voor de hand ligt, kan immunomodulatie ook een ongewenst negatief effect hebben in deze patiënten: namelijk versnelde afstoting van het transplantaat. Dit betekent dat versterking van de afweer tegen infecties tevens de patiënt in een levensbedreigende situatie kan brengen door ook de afweer tegen het transplantaat te versterken. In hoofdstuk zes wordt het effect beschreven van immunomodulatoren op huidtransplantaten van muizen. We vonden dat activatie van de afweer van deze muizen met LE-MTPPE, LE-IFN-γ of zelfs LE-MTPPE/IFN-γ geen effect had op de afstoting van de huidtransplantaten. Zowel in immunocompetente als in T cel gedepleteerde muizen, resulteerde effectieve stimulatie van de afweer niet in versnelde afstoting van de transplantaten. Daaruit concluderen wij dat immunomodulatie mogelijk is in transplantaatdragende gastheren zonder negatief effect op het transplantaat.

De positieve resultaten die verkregen werden met de immunomodulatoren zoals beschreven in hoofstuk twee, en de indicatie dat macrofagen een belangrijke rol spelen in het immunomodulatieproces, maar dat ook andere cellen van belang zijn, heeft aangezet tot een nadere bestudering van de macrofaag. Hoewel macrofagen geen verhoogde opname of doding van bacteriën laten zien na blootstelling aan immunomodulatoren in vitro, tonen in vivo experimenten aan dat depletie van macrofagen effectieve immunomodulatie compleet onmogelijk maakt. De in vitro studies die beschreven worden in hoofdstuk twee werden echter wel gedaan met peritoneaal macrofagen. Wij speculeren dat de macrofagen die betrokken zijn bij het proces volgend op immunomodulatie in vivo vooral in de lever en de milt aanwezig zijn. Daarom werden pogingen ondernomen om macrofagen uit deze organen te isoleren en hun gedrag na blootstelling aan immunomodulatoren in vitro te bestuderen. In hoofdstuk zeven wordt de isolatie van lever macrofagen (Kupffer cellen, KC) en milt macrofagen van de muis beschreven. Door middel van counterflow centrifugatie elutriatie (CCE) konden KC in grote aantallen tot hoge zuiverheid geïsoleerd worden. Tevens slaagden we erin om ook een verhoogde zuivering van miltmacrofagen te bewerkstelligen. De macrofagen overleefden de isolatie in hoge percentages en konden tot enkele weken na isolatie in vitro gekweekt worden. Zuiverheidsbepalingen door middel van specifieke antilichamen, enzymatische testen of door middel van histologie lieten zien dat er een hoge mate van correlatie bestaat tussen deze testen. Daarnaast werd aangetoond dat de zuiverheid van de macrofagenfracties ook relatief simpel bepaald konden worden door gebruik te maken

van de autofluorescentie van macrofagen en deze te meten in een flowcytometer. Gebruikmakende van de CCE techniek waren we tevens instaat om de macrofagen uit de lever en milt verder onder te verdelen op basis van grootte in drie afzonderlijke fracties. Functionaliteitstesten laten zien dat macrofagen in de verschillende fracties van elkaar verschillen in activiteit. Vooral de kleine KC vertonen een hoge zuurstof metabolisme na blootstelling aan bacteriën. Dit werd ook aangetoond voor de kleine miltmacrofagen. Stikstofoxide (NO) en TNF–α daarentegen werden vooral geproduceerd door de grote KC na blootstelling aan bacteriën. Alle KC vertoonden echter een gelijke (hoge) mate van fagocytose. Deze resultaten laten zien dat er niet alleen verschillen bestaan in activiteit tussen de verschillende macrofaag populaties (bijvoorbeeld tussen KC en miltmacrofagen), maar dat ook binnen een populatie een grote heterogeniteit bestaat. Deze bevindingen correleren in hoge mate met bevindingen van anderen, die een relatie aantoonden tussen KC grootte en lokalisatie in de lever.

In hoofdstuk acht werd de heterogeniteit van KC verder onderzocht in relatie tot immunomodulatie. Drie KC fracties (met cellen omschreven als klein, intermediair en groot) werden onderzocht op hun vermogen tot produktie van NO en TNF–α na priming met IFN–γ en/of TNF–α en een daarop volgende blootstelling aan hitte gedode bacteriën. Als reeds boven beschreven, vertonen kleine en intermediaire KC geringe tot geen TNF–α of NO produktie. Grote KC daarentegen produceren NO and TNF–α in hoge mate na blootsteling aan hitte gedode bacteriën. Priming van KC met IFN–γ voor toevoegen van bacteriën resulteerde in een toegenomen TNF–α produktie in grote KC en een toename van de NO produktie in alle KC fracties. Priming van de KC met TNF–α resulteerde in een enorme toename van de TNF–α produktie door de kleine KC, maar had daarentegen slechts gering effect op de NO produktie. Priming van de KC met de combinatie van TNF–α en IFN–γ voor blootstelling met bacteriën resulteerde vreemd genoeg in een reductie van de TNF–α produktie vergeleken met TNF–α geprimede cellen. De NO produktie werd echter gestimuleerd in alle KC fracties door de combinatie van TNF–α en IFN–γ op een synergistische wijze. Deze resultaten tonen aan dat er een hoge mate van heterogeniteit bestaat in de KC populatie welke tevens zijn weerslag vindt in een immunologische reactie van de KC. Duidelijk blijkt dat de KC verschillende functies hebben in de immuun respons die gerelateerd lijkt te zijn aan de grootte van deze cellen.

Immunomodulatie in de behandeling van een letale sepsis kan erg effectief zijn, en kan zoals door ons werd aangetoond in het experimentele infectiemodel weergegeven in dit proefschrift, resulteren in 100% overleving. In dit proces lijken macrofagen een sleutelrol te vervullen, maar ook andere cellen (bijvoorbeeld T cellen) blijken belangrijk. De studies hier beschreven tonen aan dat immunomodulatie méér inhoudt dan macrofaag activatie alleen en dat ook ander celtypen zijn betrokken. Het inzicht dat wij hebben in het mechanisme dat ten grondslag ligt aan het immunomodulatieproces wordt verder bemoeilijkt door de heterogeniteit van de sleutelcel: de macrofaag. Verdere onderzoek op dit terrein is noodzakelijk om ons begrip van de mechanismen achter de immuno-modulator-geïnduceerde afweerversterking te vergroten. Hopelijk zal de verkregen kennis leiden tot een meer succesvolle behandeling van ernstige infecties met name in immunogecompromiteerde patiënten waar de antibioticumbehandeling faalt

ABBREVIATIONS

CCE	counter current elutriation
CD	clusters of differentiation
CFU	colony forming units
CR3	complement receptor 3
CSF	colony stimulating factor
ELISA	enzyme linked immunosorbant assay
FcR	Fc receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
IFN–γ	interferon-gamma
IL	interleukin (as in IL-1, IL-2 etc)
i.p.	intraperitoneal
i.v.	intravenous
KC	Kupffer cells
LE	liposome-encapsulated (as in LE-IFN–γ and LE-MTPPE)
LPS	lipopolysaccharide
mAb	monoclonal antibody
MDP	muramyl dipeptide
MHC-II	major histocompatibility complex class II
MPS	mononuclear phagocyte system
MTPPE	muramyl tripeptide phosphatidylethanolamine
mu	murine (as in muIFN–γ)
NO	nitric oxide
PC	phosphatidylcholine
PL	placebo liposomes
PS	phosphatidylserine
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
s.c.	subcutaneous
Th	T helper cell
TNF–α	tumor necrosis facto-alpha
U	units

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PUBLICATIONS

Detoxified Ra-LPS prevents suppression by free capsular polysaccharide of the immune response towards a polysaccharide-protein conjugate.
Timo L.M. ten Hagen, André F.M. Verheul. Harm Snippe, and Robert L. Hunter.
Vaccine Res 2, 215-25,1993.

Role of adjuvants in the modulation of antibody isotype, specificity, and induction of protection by whole blood-stage *Plasmodium yoelii* vaccines.
Timo L.M. ten Hagen, Alexander J. Sulzer, Marybeth R. Kidd, Altaf A. Lal, and Robert L. Hunter.
J Immunol 151, 7077-85, 1993.

Effect of free and liposome-encapsulated MTPPE and IFN–γ on *Listeria monocytogenes* infection in mice.
Pernella M.B. Melissen, Timo L.M. ten Hagen, Wim van Vianen, Marian van Marion, and Irma A.J.M. Bakker-Woudenberg.
J Liposome Res 3, 170-72, 1993.

The role of HSV-induced Fc- and C3b(i)-receptors in bacterial adherence.
Lia A.M. de Graaf-Miltenburg, Karin E. van Vliet, Timo L.M. ten Hagen, Jan Verhoef, and Jos A.G. van Strijp.
J Med Microbiol 40, 48-54, 1994.

Effect of liposome-encapsulated muramyl tripeptide phosphatidylethanolamine and interferon gamma on *Klebsiella pneumoniae* infection in T-cell-deficient mice and in vitro
Timo L.M. ten Hagen, Wim van Vianen, Elma A.T. Straathof, and Irma A.J.M. Bakker-Woudenberg.
in: Immunotherapy of infections, ed Masihi K.N., Marcel Dekker, Inc., 225-34, 1994.

Modulation of nonspecific antimicrobial resistance of mice to *Klebsiella pneumoniae* septicemia by liposome-encapsulated muramyl tripeptide phosphatidylethanolamine and interferon–γ alone or combined
Timo L.M. ten Hagen, Wim van Vianen, and Irma A.J.M. Bakker-Woudenberg.
J Infect Dis 171, 385-92, 1995.

Liposomes as delivery systems in the prevention and treatment of infectious diseases.
Joep J. Bergers, Timo L.M. ten Hagen, Els W.M. van Etten, and Irma A.J.M. Bakker-Woudenberg.
Pharm World Sci, 17, 1-11, 1995.

Enhancement of nonspecific resistance by liposome-encapsulated immunomodulators does not affect skin graft rejection in mice.
Timo L.M. ten Hagen, Ann C.T.M. Vossen, Wim van Vianen, G. John M. Tibbe, Huub F.J. Savelkoul, Hubertine Heremans, and Irma A.J.M. Bakker-Woudenberg.
Transplantation 60, 1211-1214, 1995.

Isolation and characterization of murine Kupffer cells and splenic macrophages.
Timo L.M. ten Hagen, Wim van Vianen, and Irma A.J.M. Bakker-Woudenberg.
J Immunol Methods 193, 81-91, 1996.

Involvement of T cells in immunomodulation of mice to *Klebsiella pneumoniae* septicemia.
Timo L.M. ten Hagen, Wim van Vianen, and Irma A.J.M. Bakker-Woudenberg.

J Liposome Res. In press, 1996

Immunostimulation in vivo with liposomal MTPPE plus interferon–γ: the effect of liposomal co-encapsulated immunomodulators on cell populations in liver, spleen, blood and bone marrow of mice.
Timo L.M. ten Hagen, Pieter J.M. Leenen, Wim van Vianen, Jane S.A. Voerman, Hubertine Heremans, and Irma A.J.M. Bakker-Woudenberg.
Manuscript submitted.

Involvement of T cells in enhanced resistance to *Klebsiella pneumoniae* septicemia of mice treated with liposome-encapsulated MTPPE and IFN–γ.
Timo L.M. ten Hagen, Wim van Vianen, Huub F.J. Savelkoul, Hubertine Heremans, Wim A. Buurman, and Irma A.J.M. Bakker-Woudenberg.
Manuscript submitted.

Differential nitric oxide and TNF–α production of murine Kupffer cell subfractions upon priming with IFN–γ and TNF–α
Timo L.M. ten Hagen, Wim van Vianen, Hubertine Heremans, and Irma A.J.M. Bakker-Woudenberg.
Manuscript submitted.

Expression of immunoregulatory cytokines determined by mRNA-RT-PCR after immunomodulation using liposome-encapsulated MTPPE/IFN–γ in mice.
Timo L.M. ten Hagen, Wim van Vianen, Huub F.J. Savelkoul, Hubertine Heremans, and Irma A.J.M. Bakker-Woudenberg.
Manuscript submitted.

Immunomodulation to Gram-negative septicemia. Effectivity and mechanism of treatment with liposomal MTPPE and IFN–γ in *K. pneumoniae* infected mice.
Timo L.M. ten Hagen, and Irma A.J.M. Bakker-Woudenberg.
Manuscript on request, Review, 1996, Old Herborn University Seminar, New Antimicrobial Strategies, Hohe Schule, Herborn-Dille, Germany.

Immunomodulation with MDP analogues and IFN–γ in experimental Gram-negative septicemia: Emphasis on the use of liposomes as drug carriers.
Timo L.M. ten Hagen, Wim van Vianen, and Irma A.J.M. Bakker-Woudenberg.
Manuscript submitted as review.

Localization of TNF–α containing Stealth® liposomes in soft tissue sarcoma in rat limb tumor model.
Alexander H. van der Veen, Nico M.C Durante, Alexander M.M. Eggermont, and Timo L.M. ten Hagen.
Manuscript in preparation.

Treatment of soft tissue sarcoma in rat limb model with DOX-SL in combination with Stealth® TNF–α (TNF-SL).
Timo L.M. ten Hagen, Alexander H. van der Veen, Nico M.C Durante, and Alexander M.M. Eggermont.
Manuscript in preparation.

Treatment of solid limb tumors with liposome-encapsulated tumor necrosis factor–α in combination with liposomal encapsulated doxorubicin in the rat.
A.H. van der Veen, T.L.M. ten Hagen, S.T. van Tiel, A.L.B. Seynhaeve, and A.M.M. Eggermont.
In: Progress in Drug Delivery System. Hirota S, Okada S, Kimura R, Nozawa Y, Oku N. Eds. Vol. 5. 101-104,

1996.

Treatment of solid tumors. Rat extremity soft tissue sarcoma model for the study of systemic treatment with stealth® liposome-encapsulated tumor necrosis factor–α and cytotoxic agents.
T.L.M. ten Hagen, and A.M.M. Eggermont
In: Progress in Drug Delivery System. Hirota S, Okada S, Kimura R, Nozawa Y, Oku N. Eds. Vol. 5. 101-104, 1996.

Treatment of solid tumors. Rat extremity soft tissue sarcoma model for the study of systemic treatment with Stealth® liposome-encapsulated tumor necrosis factor–α and cytotoxic agents.
T.L.M. ten Hagen, and A.M.M. Eggermont.
Adv Drug Delivery Rev. In press, 1996.

Tumor necrosis factor α in the treatment of solid tumors: Old agent, new applications.
T.L.M. ten Hagen, and A.M.M. Eggermont.
In: Long Circulating Liposomes: Old Drugs, New Therapeutics. M.C. Woodle and G. Storm. Eds. In press, 1997.

Regional application of TNF–α in the treatment of cancer: A preclinical-clinical interactive program.
A.A.M. Eggermont, E.R. Manusama, and T.L.M. ten Hagen.
J Inflammation. In press, 1996.

ABSTRACTS

Effect of adjuvants on isotype of antibody to whole blood stage *P. yoelii*.
Timo L.M. ten Hagen, Alexander J. Sulzer, Altaf A. Lal, and Robert L. Hunter.
FASEB, Atlanta 1991, the FASEB J 5: #5762.

Effect of adjuvants on the specificity of antibody to whole blood stage *P. yoelii*.
Timo L.M. ten Hagen, Alexander J. Sulzer, Altaf A. Lal, and Robert L. Hunter.
40th annual meeting, Am Soc Tropical Med Hygiene, Boston 1991, Am J Tropical Med Hygiene 45: #116.

Adherence of bacteria to HSV-1 infected cells: role of HSV Fc-receptor and C3b-receptor.
Karin E. van Vliet, Timo L.M. ten Hagen, Lia A.M. de Graaf-Miltenburg, Jos A.G. van Strijp, and Jan Verhoef.

The effect of liposome-encapsulated immunomodulators in mice developing sepsis after i.p. challenge with *Klebsiella pneumoniae*.
Timo L.M. ten Hagen, Wim van Vianen, Elma A.T. Straathof, and Irma A.J.M. Bakker-Woudenberg.
Int Symp Immunother Infect, Berlijn 1993, Program and Abstracts: 34.

Effect of free and liposome-encapsulated MTPPE and IFN–γ on *Listeria monocytogenes* infection in mice.
Pernella M.B. Melissen, Timo L.M. ten Hagen, Wim van Vianen, Marian van Marion, and Irma A.J.M. Bakker-Woudenberg.

The Second Liposome Res Days, Leiden 1992, Abstract book: P7.

Involvement of T-cells in immunomodulation of mice to *Klebsiella pneumoniae* septicemia.
Timo L.M. ten Hagen, Wim van Vianen, and Irma A.J.M. Bakker-Woudenberg.
The Fourth Liposomes Res Days, Freiburg 1995, Abstractbook: L14.

Murine Kupffer cells. Isolation and characterization of liver macrophages.
Timo L.M. ten Hagen, Wim van Vianen, and Irma A.J.M. Bakker-Woudenberg.
The macrophage. Basic Clinical Aspects Amsterdam1995, Abstractbook P5.

Biodistribution and toxicity of liposome-encapsulated tumor necrosis factor alpha in the rat.
A.H. van der Veen, T.L.M. ten Hagen, R.L. Marquet, I.A.J.M. Bakker-Woudenberg, and A.M.M. Eggermont.
VII. International Congress on Regional Cancer Treatment, Weisbaden 1995, Abstractbook.

Biodistributie en toxiciteit van vrij en liposomaal tumor necrosis factor–α in de rat.
A.H. van der Veen, T.L.M. ten Hagen, R.L. Marquet, I.A.J.M. Bakker-Woudenberg, en A.M.M. Eggermont.
Nederlandse vereniging van heelkunde, chirurgendagen, Veldhoven 1995, Abstractboek.

Behandeling van het BN175 wekedelen sarcoma in de rat met TNF–α en doxorubicine ingekapseld in lang circulerende liposomen.
A.H. van der Veen, T.L.M. ten Hagen, N.M.C. Durante, en A.M.M. Eggermont.
Nederlandse vereniging van heelkunde, chirurgendagen, Veldhoven 1996, Abstractboek

Biodistribution of TNF–α encapsulated in long circulating liposomes in the BN175 extremity soft tissue sarcoma model in brown norway rats.
T.L.M. ten Hagen, A.H. van der Veen, R.L. Marquet, and A.M.M. Eggermont
TNF–α and related cytokines: Clinical utility and biology of action, Hilton Head Island1996, Abstractbook.

Treatment of solid limb tumors with liposome-encapsulated tumor necrosis factor α, doxorubicin and melphalan in the rat.
A.H. van der Veen, T.L.M. ten Hagen, R.L. Marquet, and A.M.M. Eggermont.
75th Annual Meeting American Assoc. Cancer Res., Washington DC 1996, Abstractbook.

Treatment of the BN 175 extremity soft tissue sarcoma in brown norway rats with TNF–α encapsulated in long circulating liposomes (LCL) in combination with LCL encapsulated cytotoxic agents.
A.H. van der Veen, T.L.M. ten Hagen, N.M.C. Durante, en A.M.M. Eggermont.
Fifth Liposome Res Days, Shizuoka 1996, Abstractbook .

Rat extremity soft tissue sarcoma model: systemic treatment of solid tumors with tumor necrosis factor–α in Stealth® liposomes in combination with DOX-SL®.
T.L.M. ten Hagen, and A.M.M. Eggermont
Fifth Liposome Res Days, Shizuoka 1996, Abstractbook .

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