# ALVEOLAR MACROPHAGE ELIMINATION IN VIVO IS ASSOCIATED WITH AN INCREASE IN PULMONARY IMMUNE RESPONSE IN MICE

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Exposure to a wide variety of antigens in ambient air takes place almost continuously via the lung. As the main part of these antigens are trivial and only the minority is potentially pathogenic, the immune response in the lung needs to be tightly controlled in order to avoid obstruction of normal lung functioning.

In addition to an effective mechanical protection in airways, such as the nasal filter, bronchial cilia, mucus, and surfactant, the respiratory tract is also protected by an elaborate immunological defense system. Large populations of lymphocytes can be found dispersed throughout lung tissue (1–4) and in specialized compartments, like bronchus-associated lymphoid tissue (5) and draining lymph nodes. In addition to this, nonlymphoid dendritic cells (1, 6, 7), bronchial and interstitial macrophages, and alveolar macrophages (AM)[1] (1, 2, 8, 9) are present in the respiratory tract.

The AM have been assigned an important role in removing antigen from the lungs by nonspecific phagocytosis, leading to transportation of these antigens, incorporated in the AM, up the mucociliary escalator out of the lung. Apart from this scavenger role, there is evidence that AM play a role in particle transportation to the lung-associated lymph nodes (LALN) (10). Furthermore, it is becoming clear that the AM can play a regulatory role in the antigen presentation by dendritic cells in lung tissue (6, 11, 12) or LALN (10, 13), as well as in the antigen-specific activation of T and B lymphocytes. Therefore, AM could perform a key role in controlling the pulmonary immune response, for which their anatomical position, strategically situated at the air-tissue interface, is also suggestive (14). Many in vitro studies with isolated AM have been performed, but they provide no unequivocal results concerning this specific function (8, 14, 15).

To study the in vivo role of AM, we adapted a method, which has been developed for the elimination of spleen macrophages (16), to eliminate AM. Using this method, we were able to remove AM from the lung of an intact animal, and it is shown that this depletion leads to a significant increase in the pulmonary immune response to an intratracheally administered antigen.

[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; AM, alveolar macrophages; $Cl_2MDP$, dichloro-methylene-diphosphonate; LALN, lung-associated lymph nodes; TNP-AF, alkaline phosphatase-coupled trinitrophenyl; TNP-KLH, trinitrophenyl-keyhole limpet hemocyanin.

## Materials and Methods

*Animals.* (C3D2)$F_1$ mice were purchased from Bomholtgård Ltd., Ry, Denmark. The mice were derived from a pathogen-free colony and were maintained in our facilities under clean conditions in isolated rooms with acidified drinking water. The mice were used in the experiments when 6–8 wk old.

*Liposomes.* The liposomes were composed of phosphatidylcholine and cholesterol (molar ratio 6:1) and contained either PBS, or dichloro-methylene-diphosphonate ($Cl_2MDP$) dissolved in PBS (0.189 g/ml). They were prepared as described before (16), in short: 86 mg phosphatidylcholine and 8 mg cholesterol were dissolved in 10 ml chloroform, and by low vacuum rotary evaporation a lipid film was produced, which was dissolved in either 4 ml PBS or 10 ml $Cl_2MDP$. The suspension was kept at room temperature for 2 h, after which it was sonicated for 3 min in a waterbath sonicator and again kept for 2 h at room temperature. The $Cl_2MDP$ liposome suspension was then diluted in 100 ml PBS and centrifuged at 100,000 *g* for 30 min to remove free $Cl_2MDP$, after which the liposomes were resuspended in 4 ml PBS.

*Administration of Liposome Suspension or Antigen.* The mice were fixed in an upright position under total anesthetization with 40 μl of a 4:3 mixture of Aescoket (Aesculaap N. V., Gent, Belgium) and Rompun (Bayer, Leverkussen, FRG), intramuscularly injected. Using a nylon tube, connected to a 1-ml syringe fixed in a micro-manipulator, 100 μl of liposome suspension or antigen was administered through the glottis into the trachea.

*Immunization Procedure.* Animals received 80 μg trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) via the trachea, as described above, dissolved in 100 μl PBS. At different intervals the animals were killed and different organs were removed and used for histocytochemistry or in plaque-forming cell assay.

*Detection of Lung Macrophages: Tissue Sections.* The lungs were removed and vacuum fixed, immersed in paraformaldehyde/lysine fixative (17), for 45 min. They were transferred to 20% sucrose, snap frozen in liquid nitrogen, and cut in 8-μm sections on a cryostat (−20°C). The sections were thereupon stained for acid phosphatase activity (18).

*Detection of Lung Macrophages: Quantification of Interstitial Lung Macrophages.* To determine the effect of the $CL_2MDP$ and PBS liposomes on the interstitial macrophage population, animals received either type of liposomes. 2 d later the animals were anesthesized with 75 μl Hypnorm (Janssen Pharmaceutica B. V., Tilburg, The Netherlands), and the thorax was opened. The pulmonary vasculature was perfused with 10 ml PBS at 37°C, containing 500 μl thromboliquine (Organon Teknika B. V., Boxtel, Holland) per liter PBS, through the right ventricle of the heart after severing the aorta. The lungs were removed if they appeared pale white and were lavaged with 0.6 mM EDTA in PBS at 37°C. The first two washings were performed with 0.5 ml lavage fluid, after which nine others followed of 1 ml each. The lungs were then minced and incubated in 10 ml RPMI 1640 containing 229 U of collagenase (Cooper-Biomedical, Inc., Malvern, PA) and 0.1 mg/ml DNase (grad. II, lot no. 10912321-30; Boehringer Mannheim FRG) for 90 min at 37°C under constant agitation. The cells were collected, gently suspended through a nylon gauze, and washed three times in RPMI 1640 containing 5% FCS. To determine the percentage of macrophages in the thus-prepared lung suspension, the cells were stained with MOMA-2, an mAb specific for tissue macrophages (19). The number of MOMA-2-positive cells was determined by analysis with a FACS (FACStar, Becton Dickinson & Co., Mountain View, CA) in combination with an FITC-labeled rabbit anti-rat $(Fab)_2$ antiserum. In addition to this, cytocentrifuge preparations were made of the lung suspensions, which were stained for acid phosphatase activity (18).

*Detection of TNP: Specific Antibody-forming Cells.* Spleen, LALN, and lungs were removed and snap frozen by immersion in liquid nitrogen, cut in 8-μm sections on a cryostat, and fixed with acetone for 10 min. The presence of TNP-specific, antibody-forming cells (AFC) was histochemically determined according to Claassen and Van Rooijen (20), using alkaline phosphatase-coupled TNP (TNP-AF) as conjugate. Alkaline phophatase activity was demonstrated using naphtol AS MX phosphate as substrate and fast blue BB base as chromogen, resulting in blue staining of TNP-specific AFC (18, 20). The slides were thereafter lightly counterstained with hematoxiline and embedded in gelatine.

*Plaque-forming Cell Assay.* The number of TNP AFC and the isotype of the produced TNP

antibodies in LALN was determined by direct (IgM) and indirect (IgG, IgA, IgE) plaque-forming cell assays (21). TNP was coupled to SRBC according to Naor et al. (22), and these cells were used as indicator cells in the plaque-forming cell assay. The isotype-specific anti-IgE, -IgA, and -IgG antisera were kindly donated by Dr. H. Savelkoul, Erasmus University Rotterdam, The Netherlands.

## Results

In mice, elimination of splenic macrophages can be accomplished by an intravenous injection with 200 $\mu$l of liposome-encapsuled $Cl_2$MDP (16). Using the same $Cl_2$MDP liposomes, we injected mice intratracheally with 100 $\mu$l liposome suspension to see if this would lead to elimination of the AM. Because of the use of vacuum fixation, we were able to make a clear distinction between alveolar and interstitial macrophages on the basis of their anatomical localization in acid phosphatase-stained lung sections. Under normal fixation conditions this distinction is hard to make, owing to the collapse of lung tissue.

When the mice were examined for the presence of AM, it was found that already within 2–3 h after a single dose of 100 $\mu$l $Cl_2$MDP liposomes, allmost all AM were depleted (Fig. 1 *B*), except for an occasional AM in the smaller lung lobes. At day 5 after administration, some AM could be found returning, but complete repopulation of the lung with AM was only reached around day 18. In contrast, animals treated with PBS liposomes showed normal AM populations throughout the period exam-

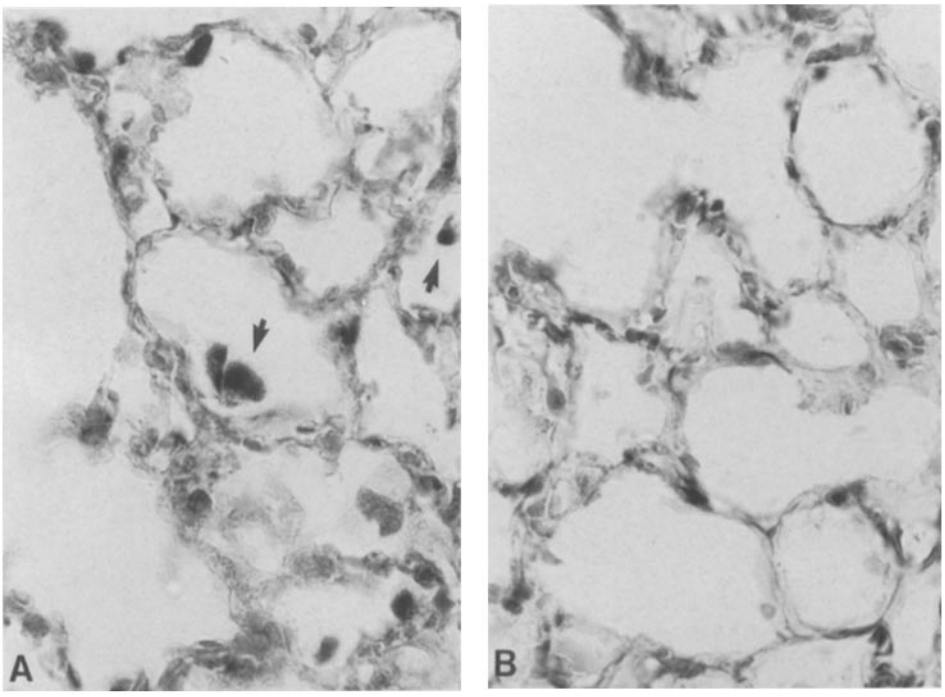


FIGURE 1. Microphotographs showing alveoli in lung of mice, stained for acid phosphatase activity. (*A*) Alveoli of a control mouse, demonstrating numerous AM (*arrows*). (*B*) Alveoli of a mouse 2 d after intratracheal injection with $Cl_2$MDP liposomes, note the absence of AM (× 40).

ined (Fig. 1 *A*). It was furthermore found that the $Cl_2MDP$ liposome treatment had no effect on the number and location of interstitial macrophages. $Cl_2MDP$ and PBS liposome-treated groups showed identical percentages of interstitial macrophages (Table I). This was supported by the acid phosphatase-stained lung sections, which displayed normal interstitial macrophage populations (compare Fig. 1, *A* and *B*). Administration of PBS liposomes in a solution of free $Cl_2MDP$ in PBS, up to 10 times the amount encapsulated in the injected liposomes, or free $Cl_2MDP$ alone in the same concentration had no effect on the alveolar or interstitial macrophage populations either (results not shown).

To study whether this elimination of AM from the lung would influence the pulmonary immune response, we immunized animals intratracheally with TNP-KLH 2 d after liposome treatment. At this timepoint, the lungs of $Cl_2MDP$ liposome-treated animals were devoid of AM (Fig. 1 *A*) and the animals were then considered to be AM-depleted animals.

The formation of specific TNP-AFC in both AM-depleted and control animals at various timepoints after immunizations is depicted in Table II. In both groups

TABLE I

*The Effect of $Cl_2MDP$ or PBS Liposome Treatment on the Interstitial Macrophage Population*

| Liposome treatment | MOMA-2 | AP |
|---|---|---|
| $Cl_2MDP$ | 3.36 (± 0.56) | 3.49 (± 0.86) |
| PBS | 3.07 (± 0.74) | 2.85 (± 0.47) |

Indicated are the mean percentages of positive cells (± SEM) in lung suspensions from five individual animals per group, as determined by FACS analysis of MOMA-2 staining, or countings of cytocentrifuge preparations stained for acid phosphatase activity. AP, acid phosphatase activity; $Cl_2MDP$: $Cl_2MDP$/liposome-treated animals; PBS: PBS/liposome-treated animals.

TABLE II

*The Effect of AM Elimination on the Pulmonary Immune Response*

| Days after immunization | Lung | | LALN | | Spleen | |
|---|---|---|---|---|---|---|
| | $Cl_2MDP$ | PBS | $Cl_2MDP$ | PBS | $Cl_2MDP$ | PBS |
| 4 | – | – | – | – | – | – |
| 6 | – | – | + + + | + | – | – |
| 8 | +/– | – | + + + - | | | |
| | | | + | + + | – | – |
| 11 | + + | – | + + | +/– | – | – |
| 13 | + | – | + | – | – | – |
| 15 | + | – | + | – | – | – |
| 28 | + | ND | +/– | ND | ND | ND |

At several intervals after intratracheal administration of TNP-KLH, the number of TNP-specific AFC was determined in a semi-quantitative manner. Each group contained two to four animals. The response is represented as the number of AFC per section of either lung, lung-associated lymph node, or spleen. No AFC detectable, –, 1-10, +/–, 10-50, +, 50-100, + +, 100-200, + + +, >200 AFC per section, + + + +. $Cl_2MDP$, $Cl_2MDP$/liposomes-treated animals; PBS, PBS/liposomes-treated animals.

AFC are primarily found in the LALN. It is conspicuous, however, that compared with the few AFC found in control mice, the number of TNP-specific AFC in LALN of AM-depleted mice has increased dramatically (compare Fig. 2, *A* and *B*). In addition, the immune response in LALN of AM-depleted mice is also longer lasting than that in control mice, and even on day 28 after immunization, TNP-specific AFC can still be found.

In control mice, specific AFC can be found in LALN, albeit in low numbers, but there is no accumulation of TNP-specific AFC in the lungs of these animals for the

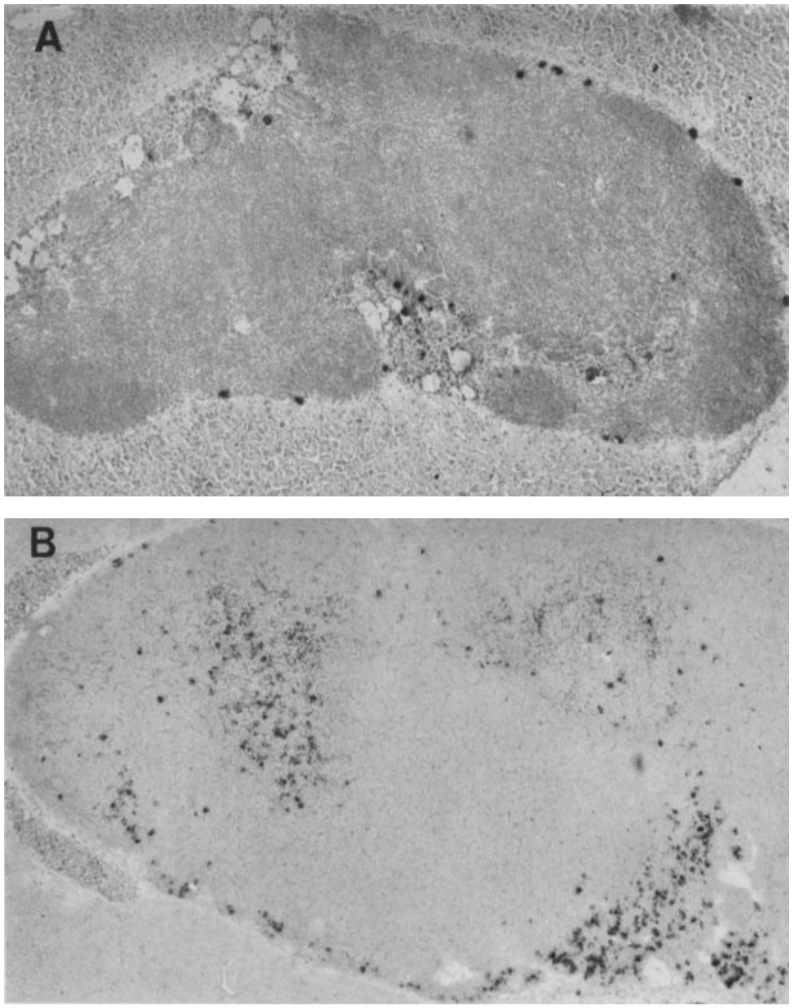


FIGURE 2. Microphotographs from LALN of mice, intratracheally immunized with 80 μg TNP-KLH 2 d after liposome treatment, stained to reveal TNP-specific AFC, and lightly counterstained with hematoxiline (× 10). (*A*) LALN of a control mouse, 8 d after immunization, displaying few TNP-specific AFC, mainly located in the medulla. (*B*) Numerous TNP-specific AFC that are found more dispersed throughout the LALN of an AM-depleted mouse, 8 d after immunization.

period tested (Fig. 3 *A*). In contrast, numerous TNP-specific AFC can be found in infiltrates around blood vessels in the AM-depleted animals. The first are found on day 8 around large blood vessels (Fig. 3 *B*), while from day 13, TNP-specific AFC are found more dispersed throughout the lung. In spleen no response is found at any timepoint, nor in the AM-depleted, nor in the control animals.

The plaque-forming cell assays performed with lymphocyte suspensions of the LALN clearly confirm these results. A dramatic increase in number of PFC is found in LALN of AM-depleted animals compared with control animals (Fig. 4). The re-

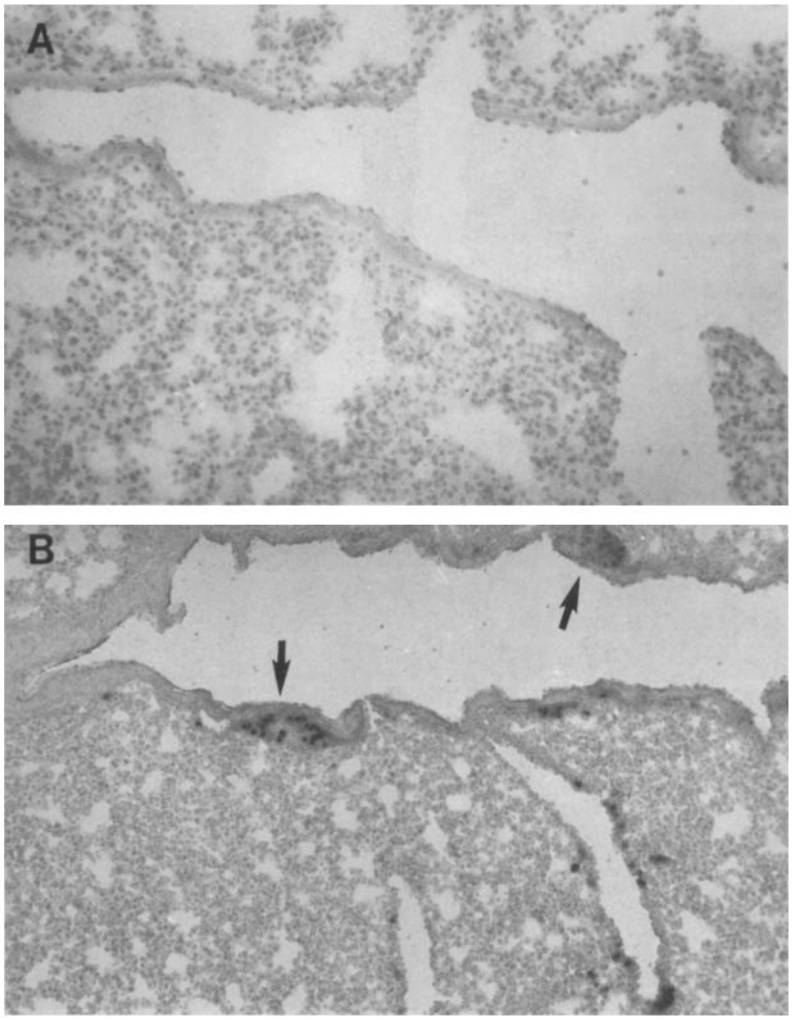


FIGURE 3. Microphotographs from lung of mice intratracheally immunized with 80 μg TNP-KLH 2 d after liposome treatment, stained to reveal TNP-specific AFC, and lightly counterstained with hematoxiline (× 10). (*A*) Lung from a control mouse, 11 d after immunization, in which no TNP-specific AFC can be demonstrated (× 1.6). (*B*) Lung of an AM-depleted mouse, 11 d after immunization; note the numerous TNP-specific AFC (*arrows*) situated in infiltrates around the major blood vessels.

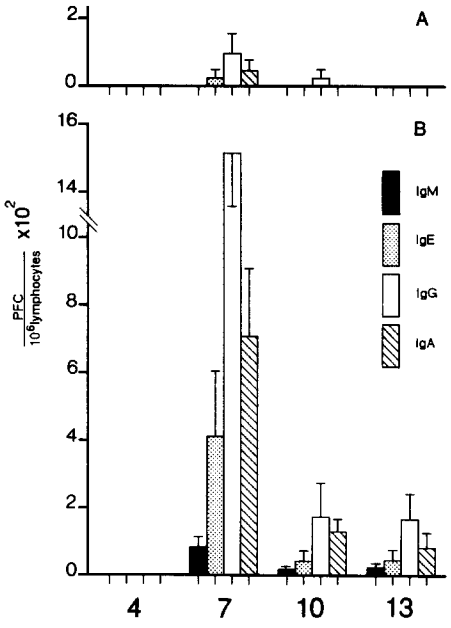


FIGURE 4. Kinetics of appearance of TNP-KLH-specific AFC in LALN, 4, 7, 10, and 13 d after intratracheal administration of 80 μg TNP-KLH. 2 d before immunization, animals were pretreated with PBS liposomes (*A*) or $Cl_2MDP$ liposomes (*B*). Numbers of AFC were determined using direct (IgM) and indirect (IgG, IgA, IgE) plaque-forming cell assay (23), using TNP-SRBCs as indicator cells (24). Data points represent mean values (± SEM) and each data point represents three experiments.

sponse in the AM-depleted and control mice show the same kinetics and there seems to be no change in isotype specificity between the two groups. The majority of the antibodies in both experimental groups consist of IgG, followed by IgA, and IgE, and very little IgM antibodies are found. Again, as already seen in the histochemical demonstration of AFC, virtualy no PFC were found in the spleen of these animals (results not shown).

## Discussion

The results of the elimination experiments clearly show that liposome-encapsuled $Cl_2MDP$ is capable of eliminating AM when injected intratracheally. The $Cl_2MDP$ liposomes are phagocytized, and after digestion of the liposomal membranes, the $Cl_2MDP$ is released into the cell. This leads to the rapid death of the cell by a mechanism that is still obscure, but may be related to the calcium-binding activity of the molecule or the presence of chloride ions. Liposome-encapsuled $Cl_2MDP$ thus selectively kills actively phagocytizing macrophages. It is evident that the liposomes themselves have no influence on the AM population and that free $Cl_2MDP$, which might leak out of the liposomes, has no determinable effect either. In addition to this, it was established that lung suspensions of both $Cl_2MDP$ and PBS liposome-treated animals contained the same percentage of macrophages as found by others in nonliposome-treated mice (23), so the interstitial macrophage population remains unaffected after the elimination procedure.

From these results the conclusion can be drawn that intratracheal administration of $Cl_2MDP$ liposomes results in the specific elimination of AM from the lung in mice, creating a test system that can be used to study the in vivo effect of AM.

In concordance with other experiments concerning lung immunization (24–26),

the control animals only showed a low immune response in LALN after intratracheal immunization. The outcome of the immunization experiments with AM-depleted animals, in which a drastic increase in AFC in LALN and an introduction of TNP-AFC in lung is observed, compared with the control animals, points to a suppressive effect of the AM on the pulmonary immune response in vivo.

The strongly enhanced pulmonary immune response, found after AM elimination, can be explained in several ways. To some extent it can be accounted for by a diminished antigen clearance from the lung, normally performed by AM. However, no significant increase in response was found after increasing the antigen dose up to 10-fold in control animals (results not shown).

The adjuvant effect of liposomes has been described previously in immune responses against liposome-associated antigens, i.e., antigens encapsulated in the aqueous compartment or exposed on the surface of the liposomes (27). Such liposome-adjuvant action cannot explain the increase of the pulmonary immune response since liposomes and antigen were administered with a 2-d interval and, more importantly, control animals were always treated with PBS liposomes.

Another possibility that might underly the observed increase is the release of amplifying cytokines in the alveolar space from the perishing macrophages after $Cl_2$MDP treatment. Although this may play a part, AM have been reported to produce predominantly suppressive factors, such as prostaglandine $E_2$ and only relatively small amounts of positive factors, like IL-1 (15, 28).

It is therefore more likely that the increase in response is caused by the lack of a regulatory, suppressive role of the AM. It has been demonstrated that the accessory activity of lung-derived dendritic cells in T cell activation, both in sodium periodate–induced proliferation (12), as well as in response to antigen (6, 11), can be suppressed by endogenous macrophages. This suppression is exercised by excretion of soluble "suppressive" factors, like prostaglandines, or direct cell-cell contact (6, 14). Therefore, when such a suppressive effect by endogenous macrophages is abrogated, as may be the case after AM elimination by $Cl_2$MDP liposomes, this could result in an enhanced presentation of invading antigens and enhanced T cell activation, leading to an increase in pulmonary immune response, as seen in our experiments.

For a proper initiation of the pulmonary immune response, it is at present considered necessary that the antigen should be transported to the LALN (24). Although such transportation function has been established for AM (10, 13), it could well be that other cells involved in transportation or the actual presentation, like neutrophils (29) and possibly dendritic cells (6, 7), are regulated by AM. The here-found increase in response after AM depletion would, in the scope of this theory, point to either an AM-mediated suppression of antigen presentation of the other celltypes involved in transportation, or an evocation of a suppressor mechanism, possibly involving T cells, by the AM under normal physiological conditions. Whether AM play a role in the induction of Ts cells has not yet formally been demonstrated. That such antigen- and isotype-specific Ts cells can arise in LALN of rats has been demonstrated after repeated aerosol antigen exposure (30). It may now be feasible to study the role of AM in this induction in AM-depleted mice.

The results presented here demonstrate that the presence of AM may be an important prerequisite for the downregulation of immune responses in the lung-LALN

system under normal, steady-state conditions. Perturbation of the function of AM may result in vigorous pulmonary immune responses that affect the normal physiology and function of the lung.

The exact mode of operation of AM in controlling the pulmonary immune response remains to be revealed. The model of AM-depleted mice as presented in this paper may be of use in solving this problem.

## Summary

A single intratracheal dose of liposome-encapsuled dichloro-methylene-diphosphonate resulted in the elimination of alveolar macrophages (AM) from the lung, creating a model to study the in vivo role of AM in the pulmonary immune response.

Using intratracheally administered trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH), the kinetics of the response, the location and number of TNP-specific antibody-forming cells, and the different Ig classes of the antibodies produced were studied in AM-depleted animals.

The results show that AM elimination has a dramatic effect on the pulmonary immune responses against TNP-KLH. An increase in APC in lung-associated lymph nodes and a prolongation of the response is found, as well as an introduction of APC in lung tissue. In both experimental groups, the majority of the TNP-specific antibodies produced was IgG, followed by IgA and IgE, while very few IgM antibodies could be detected.

We conclude from these results that AM are likely to play a role in controlling the pulmonary immune response in a suppressive way, thereby limiting the possible damage caused by severe immune responses in lung tissue.

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