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Alveolar Macrophages Regulate the Induction of Primary Cytotoxic T-Lymphocyte Responses during Influenza Virus Infection

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Virus-specific cytotoxic T lymphocytes (CTL) are thought to be responsible for the eradication of respiratory influenza virus infections by direct cytolysis of virus-infected epithelial cells. In this study, we provide evidence for a role for alveolar macrophages (AM) in the regulation of pulmonary virus-specific CTL responses. Prior to infection with influenza virus, AM were selectively eliminated in vivo with a liposome-mediated depletion technique, and virus-specific CTL activities of lung and mediastinal lymph node (MLN) cells were assayed ex vivo and compared with those for normal mice. AM depletion resulted in increased primary CTL responses and changed the kinetics of the CTL response. Flow cytometric analysis of lung and MLN cells showed that the percentage of $CD8^+$ cells was not altered after AM depletion and that lung cells from AM-depleted mice had an increased capacity to lyse virus-infected cells. Upon restimulation in vitro, virus-specific CTL activity in lung cells of normal mice was similar to that in lung cells of AM-depleted mice. Furthermore, elimination of AM resulted in increased virus titers in the lung, but virus clearance as a function of time was not affected. Our results show that AM regulate virus-specific CTL responses during respiratory influenza virus infection by removing viral particles, by downregulating the priming and activity of CTL in MLN cells, and by inhibiting the expansion of virus-specific CTL in the lung.

The mucosal surface of the respiratory tract is continuously exposed to the external environment and, as a result, environmental antigens, such as pathogenic microorganisms and allergens, enter the lung. Whereas a first line of defense against these potentially harmful substances is provided by the physical and chemical properties of the epithelial cells lining the respiratory tract, a complex respiratory immune system has evolved for the generation of protective immune responses. The induction of functional specific immune responses against environmental antigens is thought to occur in the lymph nodes draining the respiratory tract, where professional antigen-presenting cells (APC) interact with T and B lymphocytes (5, 14). Within the respiratory immune system, both pulmonary macrophages and dendritic cells (DC) are able to phagocytose, process, and present particulate antigens. However, DC are regarded as the main APC in vivo, due to their unique ability to stimulate antigen-specific T lymphocytes (25). The discovery of an extensive network of DC within the upper and lower respiratory tracts, similar to that of Langerhans cells in the skin (21, 23, 24), supports the hypothesis that pulmonary DC are responsible for the initiation of specific immune responses against airborne antigens. On the other hand, the pulmonary macrophage population, which consists of alveolar macrophages (AM) residing at the luminal surface in the alveoli and alveolar ducts and interstitial macrophages (IM) present within the parenchymal lung interstitium (9), may be involved in innate immune responses to invading bacteria and viruses.

Importantly, respiratory immune responses need to be tightly regulated to avoid the development of chronic inflammation and damage to the delicate blood-tissue barrier, which may interfere with the exchange of oxygen and CO_2. In addition, downregulation of local immune responses may prevent sensitization to allergens and thereby prevent diseases such as allergy and asthma. A number of studies have provided evidence that AM are involved in the suppression of respiratory immune responses by suppressing T-lymphocyte proliferation and by downregulating the accessory cell functions of lung DC, thereby suppressing their capacity to induce antigen-specific T lymphocytes (17, 18, 28). Although this immunosuppressive activity of AM may be of major importance for the control of normal lung homeostasis, the induction of adequate specific immune responses during infection with pathogenic microorganisms may require the temporary reversal of the immunosuppression to allow rapid clearance and recovery from infection. Only a limited number of reports describe the role of AM in bacterial pneumonia (10), and not much is known regarding the regulatory role of AM during virus-induced lung inflammation. The aim of the present study, therefore, was to determine the role of AM in the induction and regulation of specific immune responses during experimental respiratory virus infection.

Influenza A virus infection is airborne and is primarily an infection of the upper respiratory tract. During infection, virus spreads to the lower respiratory tract and may result in primary viral pneumonia. Influenza A virus infection is a lytic infection and causes the breakdown of the blood-tissue barrier early in infection, resulting in the influx of macrophages, neutrophils, and NK cells prior to the influx of virus-specific T lymphocytes (1). Cytotoxic T lymphocytes (CTL) can be detected in the lung 6 to 7 days after infection with influenza A virus and are thought to be responsible for clearance of the virus by direct cytolysis of virus-infected cells (11, 20, 34). The experiments described in this paper investigated the role of AM in the induction and regulation of influenza virus-specific CTL responses by comparing primary CTL responses in lung cell

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suspensions from normal and AM-depleted mice at different times after viral infection. We were able to show that elimination of AM before infection with influenza A virus not only increased virus-specific CTL activities but also induced a shift in the kinetics of the virus-specific CTL response. The results of this study provide evidence that AM may not only suppress virus-specific CTL responses by removal of viral particles but also downregulate the priming and activity of virus-specific CTL.

MATERIALS AND METHODS

Mice. Specific-pathogen-free male BALB/c mice were bred in the animal care facility of the Department of Microbiology and Immunology, University of Melbourne, and used at 6 to 8 weeks of age. Mice were kept under conventional housing, with free access to commercial mouse food and tap water.

Virus. Mice were infected with Mem71, a reassortant influenza A virus bearing the hemagglutinin of A/Memphis/1/71 (H3) and the neuraminidase of A/Bellamy/42 (N1) (kindly provided by L. Brown, Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria, Australia). The virus was grown in the allantoic cavity of 10-day-old embryonated hen eggs and harvested and stored as described previously (2). Mice were infected intranasally (i.n.) under methoxyflurane (Penthrane) anesthesia with $10^{4.5}$ PFU of influenza A virus in 50 μl of phosphate-buffered saline (PBS).

Depletion of AM in vivo. The liposome-mediated macrophage depletion technique is based on the intracellular delivery of the drug dichloromethylene diphosphonate (Cl_2MDP). Preparation of Cl_2MDP-liposomes and applications of the technique have been described in detail elsewhere (33). AM were depleted by i.n. administration under methoxyflurane anesthesia of 50 μl of Cl_2MDP-liposomes on days 4 and 2 before infection with influenza A virus (Cl_2MDP was a kind gift of Boehringer GmbH, Mannheim, Germany). As a control, mice received 50 μl of PBS-liposomes or PBS. Depletion of AM was confirmed by histologic examination (results not shown).

Preparation of single-cell suspensions from lung and MLN cells. Mice were sacrificed 6 days after infection with influenza A virus by cervical dislocation. Lungs and mediastinal lymph nodes (MLN) were aseptically removed, and single-cell suspensions were prepared as follows. Lungs from groups of five mice were pooled, and the tissue was minced and incubated for 45 min at 37°C on a rocker with 200 μg of collagenase D and 40 μg of DNase I (both from Boehringer GmbH) per ml in 10 ml of RPMI 1640 (Gibco BRL, Grand Island, N.Y.) supplemented with antibiotics, 2 mM glutamine, and 2×10^{-5} M β-mercaptoethanol (RPMI). Subsequently, the enzyme-digested lung tissue was passed through stainless steel sieves to obtain single-cell suspensions, and erythrocytes were lysed by treatment with NH_4Cl-Tris buffer. Single-cell suspensions were washed twice in RPMI, the cells were counted and viability was determined by trypan blue exclusion (viability, >90%). Any particulate matter was removed from the single-cell suspensions, which were used directly as effector cells in a standard cytotoxicity assay. In some experiments, macrophages and other adherent cells were removed from the lung cell suspensions by adherence to tissue culture flasks (Nunc, Roskilde, Denmark) for 2 h at 37°C in 5% CO_2. MLN from groups of five mice were gently passed through nylon gauze to obtain single-cell suspensions. Cells were washed twice in RPMI after depletion of erythrocytes with NH_4Cl-Tris buffer, and viable cells were counted by trypan blue exclusion (>95% viable).

Restimulation of virus-specific CTL in vitro. Single-cell suspensions obtained from enzyme-digested lungs were cultured for 5 days with Mem71-infected syngeneic splenocytes (ratio, 10:1) in 10 ml of RPMI supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO_2. Subsequently, viable cells were collected and used as effector cells in a standard cytotoxicity assay.

Cytotoxicity assay. Virus-specific cytolytic activity in single-cell suspensions from lungs or MLN of influenza A virus-infected mice was assayed in a standard ^{51}Cr release assay in which ^{51}Cr-labeled P815 cells (a mastocytoma cell line, *H-2*^{d}) were used as target cells. Prior to being labeled with 200 μCi of $Na_2{}^{51}CrO_4$ (Amersham Corp., Sydney, New South Wales, Australia), P815 cells were infected with Mem71 by incubation for 1 h at 37°C with $10^{7.5}$ PFU of Mem71 or incubated with RPMI as a control. Serial dilutions of effector cells were incubated in triplicate cultures with either noninfected or Mem71-infected target cells in 150 μl of RPMI–10% FCS in round-bottom 96-well plates at 37°C in 5% CO_2. After 5 h, 100 μl of supernatant was assayed for ^{51}Cr release. Results are presented as percent specific lysis, defined as (experimental lysis − spontaneous lysis)/(total detergent lysis − spontaneous lysis). Maximum spontaneous release values were always <10% of total lysis.

Immunofluorescence staining of cells and flow cytometry. Cells (10^6) were incubated for 30 min on ice with 25 μl of either rat anti-mouse CD4 (MT4) (22) or rat anti-mouse CD8 (Silenus, Hawthorn, Victoria, Australia) diluted with ice-cold PBS–1% FCS. After three washes with PBS–1% FCS, cells were resuspended in fluorescein isothiocyanate-conjugated rabbit anti-rat immunoglobulin (Silenus) diluted with PBS–1% FCS and incubated for 30 min in the dark on ice. As a negative control, cells were incubated with the fluorescein isothiocyanate-conjugated antibody only. Cells were washed again, resuspended in PBS–1% FCS, and immediately used for flow cytometric analysis. Prior to analysis, propidium iodide (1 μg/ml) was added to the cells to allow dead cells to be distinguished from viable cells. Cell suspensions were analyzed with a FACScan (Becton Dickinson, Mountain View, Calif.) and the CellQuest software program. Data was collected for 10,000 viable cells selected by forward and side scatter and propidium iodide uptake.

Virus titration by plaque-forming assays. Mice were infected i.n. with $10^{4.5}$ PFU of Mem71. At days 2, 4, and 6 after infection, lungs were removed, ground, and frozen in aliquots at −70°C. Virus yield in lung homogenates was quantified by a virus plaque-forming assay on Madin-Darby canine kidney (MDCK) cells. Briefly, monolayers of MDCK cells in 6-well plates were incubated with 100 μl of serial 10-fold dilutions of lung homogenates in RPMI 1640 supplemented with antibiotics for 45 min at 37°C in 5% CO_2. Subsequently, 3 ml of Leibovitz L-15 medium (Gibco BRL) supplemented with antibiotics, 0.01 M HEPES, 0.1% trypsin (treated with tolylsulfonyl phenyalanyl chloromethyl ketone [TPCK; Worthington), and 0.09% agarose (ICN Biomedicals, Sydney, New South Wales, Australia) was added to the cells, and the plates were incubated for 3 days at 37°C in 5% CO_2. Plaques were then counted. Each sample was tested in duplicate. Results are presented as mean ± standard deviation PFU/lung for groups of five mice.

RESULTS

In vivo elimination of AM with a liposome-mediated depletion technique. In this study, we used the liposome-mediated macrophage depletion technique to eliminate AM in vivo. This technique is specific for macrophages and is based on the liposome-mediated intracellular delivery of Cl_2MDP (33). Accumulation of Cl_2MDP in the cytosol damages the macrophages, resulting in their death and depletion from the tissues. A previous study showed that AM can be removed from the lungs of mice by a single intratracheal injection of 100 μl of Cl_2MDP-liposomes, without affecting neighboring cells or inducing a cellular infiltrate (29). However, since mice were infected i.n. with influenza A virus, we administered the liposomes via the same route. To effectively eliminate the AM population, mice received 50 μl of Cl_2MDP-liposomes on days 4 and 2 before infection with influenza A virus. To control for an effect of the liposome treatment itself, mice received the same amount of PBS-liposomes. This protocol resulted in the complete depletion of AM from the lungs, as determined by histologic examination on day 0, and was used for all experiments (results not shown).

Effect of AM depletion on influenza virus-specific CTL responses in the lungs. To investigate the involvement of AM in the induction of CTL responses during influenza A virus infection, groups of five normal and AM-depleted mice were infected with influenza A virus Mem71. Six days later, single-cell suspensions prepared from the lungs of these mice were tested for their ability to lyse virus-infected cells in a standard ^{51}Cr release assay. This method allowed us to study the primary CTL activity of the isolated lymphocytes without the need for a 5-day culture for expansion and therefore provides an actual quantitation of the CTL activity in the lungs. Figure 1 shows the results of a representative experiment. In normal mice, 25% specific lysis of Mem71-infected target cells was measured in cell suspensions from the lungs at an effector cell/target cell ratio (E/T ratio) of 100:1, and this activity gradually decreased at lower E/T ratios. CTL activity in single-cell suspensions obtained from AM-depleted lungs was significantly increased over that in normal mice, and maximal lysis of 95% was observed at an E/T ratio of 100:1. Importantly, virus-specific lysis was similar in cell suspensions from normal (PBS-treated) and PBS-liposome-treated mice, indicating that the increased CTL activity in AM-depleted mice was not the result of the liposome treatment per se. Since AM have been shown to suppress T-cell activity in vitro, we had to exclude the possibility that the lower CTL activity in cell suspensions from normal mice was the result of the presence of AM in vitro in the ^{51}Cr release assay. To do so, macrophages were removed

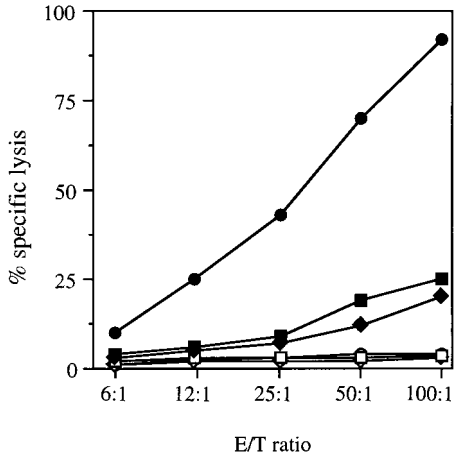

FIG. 1. Depletion of AM results in increased primary virus-specific CTL responses in the lungs. Groups of five mice were treated with PBS (■), PBS-liposomes (◆), or Cl_2MDP-liposomes (●) before i.n. infection with $10^{4.5}$ PFU of Mem71 virus. Six days after infection, single-cell suspensions prepared from pooled enzyme-digested lungs were tested for CTL activity in a 5-h ^{51}Cr release assay against Mem71-infected (closed symbols) and noninfected (open symbols) target cells.

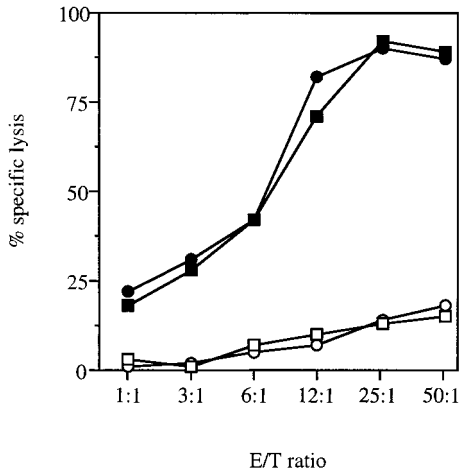

FIG. 3. Virus-specific CTL activity in in vitro-restimulated lung cell cultures. Lung cell suspensions from normal (■) and AM-depleted (●) mice were restimulated in vitro with Mem71-infected syngeneic splenocytes as APC for 5 days, after which the virus-specific CTL activity of viable cells was assayed in a ^{51}Cr release assay. Open symbols, specific lysis of noninfected target cells; closed symbols, lysis of Mem71-infected target cells.

zrom half of the single-cell suspension by allowing them to adhere to plastic tissue culture flasks for 2 h at 37°C in a humidified CO_2 incubator. After 2 h, nonadherent cells were collected; virus-specific CTL activity was measured in a ^{51}Cr release assay and compared with the CTL activity measured in the other half of the isolated cells, which were not depleted of macrophages in vitro. As is evident from Fig. 2, the lower virus-specific CTL activity in normal mice was not the result of the presence of suppressing macrophages in vitro, since CTL activity in the nonadherent cells was equivalent to the virus-specific lysis measured in the non-macrophage-depleted cell suspensions.

To determine whether the decreased cytolytic activity of virus-specific CTL in normal mice compared with that in Cl_2MDP-liposome-treated mice was permanent or was only a local pulmonary effect, lung cell suspensions were restimulated in vitro with virus-infected syngeneic splenocytes for 5 days and subsequently assayed for their ability to lyse virus-infected target cells in a cytotoxicity assay. As can be seen in Fig. 3, virus-specific CTL responses were comparable between cultures from normal and Cl_2MDP-liposome-treated mice, suggesting that the cytolytic activity of CTL derived from normal mice was only temporarily lower than that of CTL obtained from AM-depleted mice. In addition, the removal of AM did not affect virus-specific CTL activities at sites distal from the lungs, since virus-specific lysis by spleen cells was similar in cultures from normal and AM-depleted mice (results not shown).

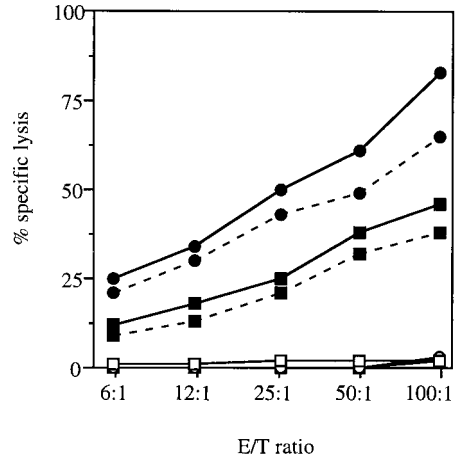

FIG. 2. Effect of in vitro elimination of macrophages on virus-specific CTL responses. Single-cell suspensions were prepared from pools of lungs from five PBS-treated (■) or Cl_2MDP-liposome-treated (●) mice infected with $10^{4.5}$ PFU of Mem71 6 days earlier. Half of the cell suspensions were incubated for 2 h in tissue culture flasks at 37°C in 5% CO_2 to remove macrophages by adherence. Subsequently, nonadherent cells were used as effector cells in a cytotoxicity assay (broken lines) and compared with the other half of the cell suspensions (solid lines). Open symbols, specific lysis of noninfected target cells; closed symbols, lysis of Mem71-infected target cells.

Effect of depletion of AM on cell number, phenotype, and virus-specific CTL activity of lung cell suspensions. Alternatively, increased CTL activity measured in a ^{51}Cr release assay may be the result of increased numbers of $CD8^+$ T lymphocytes. Therefore, the effect of in vivo elimination of AM on the cell number and phenotype of lung cell suspensions was analyzed on days 4, 5, and 6 after infection. Treatment of mice with PBS-liposomes or Cl_2MDP-liposomes resulted in an increase in the total cell number in the lungs compared with that in PBS-treated mice; this increase decreased over time after treatment (Fig. 4). Flow cytometric analysis showed that a high percentage of these cells were polymorphonuclear leukocytes (results not shown). Importantly, the percentages of $CD4^+$ and $CD8^+$ cells were not affected by either PBS-liposome or Cl_2MDP-liposome treatment relative to the levels in normal mice. Approximately 35 to 40% of cells obtained from the lungs 4 days after infection with Mem71 were $CD4^+$, and moderate increases in the percentages of $CD4^+$ cells were observed on days 5 and 6 after infection. In this particular experiment, the percentage of $CD4^+$ cells in PBS-liposome-treated mice was slightly lower than those in normal and Cl_2MDP-liposome-treated mice; however, this difference was not observed in other experiments. The percentage of $CD8^+$ cells increased from 15 to 17% on day 4 after infection to 29 to 32% 6 days after inoculation of Mem71. It is of major importance for the interpretation of the results presented in Fig. 1 and 2 (see also Fig. 6) to note that the percentages of $CD8^+$ cells in lung cell

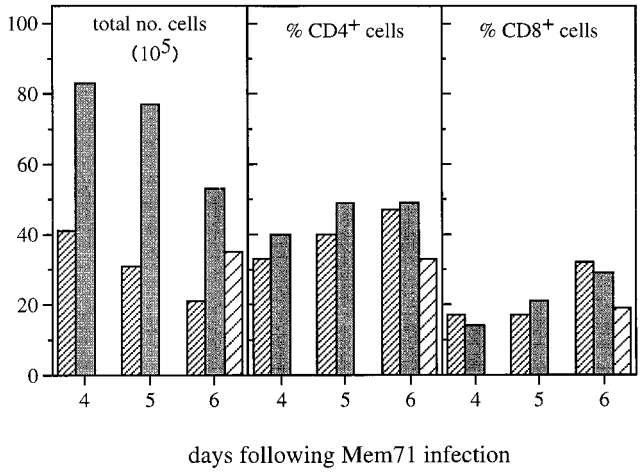

FIG. 4. Characterization of lung cell suspensions at various times after influenza virus infection. Lung cell suspensions from PBS-treated (▨), PBS-liposome-treated (▨), and Cl_2MDP-liposome-treated (■) mice were analyzed for total cell number and expression of CD4 and CD8 by flow cytometry. The results are representative of multiple experiments.

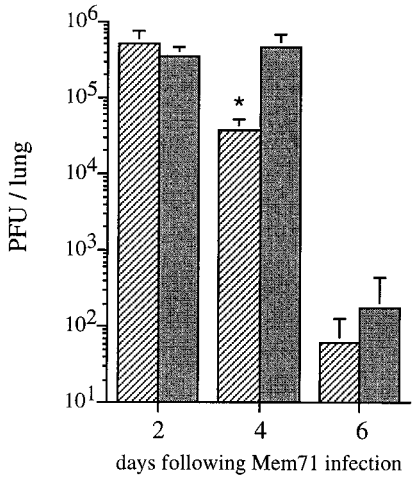

FIG. 5. Effect of AM depletion on influenza virus titers in the lungs. Groups of five PBS-treated (▨) or Cl_2MDP-liposome-treated (■) mice were sacrificed at the indicated times after infection with $10^{4.5}$ PFU of Mem71, and the virus titers in the lungs were determined by a plaque-forming assay. Represented are the results of one of two experiments. Asterisk, statistically significant difference (Student's t test; $P < 0.001$).

suspensions obtained from normal and AM-depleted mice were the same. Therefore, equivalent numbers of $CD8^+$ cells were assayed for CTL activity in the ^{51}Cr release assays, since these assays are performed with a standardized number of effector lymphocytes. Of course, the in vivo situation is different because an increased total number of cells was observed in the lungs of liposome-treated mice, meaning that an increased number of $CD8^+$ cells was present in the lungs. For example, on day 6 after infection, 1.5×10^6 $CD8^+$ cells (29% of 5.3×10^6) were isolated from the lungs of AM-depleted mice, whereas only 5.8×10^5 $CD8^+$ cells (28% of 2.1×10^6) were present in the lungs of PBS-treated mice.

To further investigate the effect of AM depletion on the induction of influenza virus-specific CTL in vivo, we analyzed whether AM depletion resulted in qualitative differences in the CTL activity of the $CD8^+$ cells. To do so, the percentage of virus-specific lysis on a per-$CD8^+$-cell basis was calculated (Table 1). This arbitrary analysis showed that 10^5 cells from lung cell suspensions obtained from AM-depleted mice had a significantly increased ability over time to lyse virus-infected cells ex vivo compared with the same number of lung cells from normal mice.

Effect of AM depletion on virus titers in the lungs. Since pulmonary macrophages may be involved in innate immune responses against viral infections, depletion of AM in vivo may affect both virus titers in the lungs and clearance of the virus. To investigate this hypothesis, virus titers in the lungs of normal and Cl_2MDP-liposome-treated mice were measured over time after infection with $10^{4.5}$ PFU of Mem71. In normal mice, virus titers were high 2 days after infection (Fig. 5) but gradually decreased, and no virus was detectable in lung homogenates 7 days after infection, indicating that the viral infection had been successfully cleared. Comparison of virus titers in the lungs of Cl_2MDP-liposome-treated mice with virus titers in the lungs of PBS-treated mice revealed the following. First, 4 days after inoculation with virus, a 10-fold increase ($P < 0.001$) in the amount of Mem71 was measured in lung homogenates of Cl_2MDP-liposome-treated mice. However, virus clearance was not affected by the elimination of AM before influenza A virus infection, since 6 days after infection, the virus loads in Cl_2MDP-liposome-treated and PBS-treated mice were comparable. In addition, clearance of virus was complete 7 days after infection in AM-depleted mice as well. Virus yield in lungs from PBS-liposome-treated mice was comparable to that in PBS-treated mice (data not shown), suggesting that the liposome treatment itself did not increase influenza A virus titers in the lungs.

TABLE 1. Effect of AM depletion on cytolytic activity of virus-specific CTL

Pretreatment	Days after infection	%		% Lysis/ $CD8^+$ cells[b]
		$CD8^+$ cells	Lysis[a]	
PBS	4	17	5	6
Cl_2MDP-liposomes	4	14	3	3
PBS	5	17	8	9
Cl_2MDP-liposomes	5	21	39	37
PBS	6	32	17	10
Cl_2MDP-liposomes	6	29	71	49

[a] Percent virus-specific lysis at an E/T ratio of 100:1.

[b] Percent virus-specific lysis per 10^5 $CD8^+$ cells was calculated with the formula [percent lysis/(percent $CD8^+$ cells × number of cells in ^{51}Cr release assay)] × 10^5, in which percent lysis represents percent virus-specific lysis at an E/T ratio of 100:1.

Effect of AM depletion on the kinetics of the virus-specific CTL response. The influence of AM elimination on the kinetics of the influenza A virus-specific CTL response in the lungs was determined by assaying CTL activity on days 4, 5, and 6 after infection. Figure 6A shows how AM depletion altered the virus-specific CTL response over time. Primary CTL activity could not be detected in normal mice on day 5 after infection, whereas lung cell suspensions from Cl_2MDP-liposome-treated mice were able to lyse target cells in a virus-specific manner.

Effect of increased virus titers on virus-specific CTL responses. Since Cl_2MDP-liposome treatment resulted in increased virus titers in the lungs during infection, the possibility that the enhanced virus-specific CTL response in AM-depleted mice resulted from exposure to a higher viral antigen load could not be excluded. The following experiment was performed to address the question of whether higher virus titers would result in higher CTL activities and changes in the kinet-

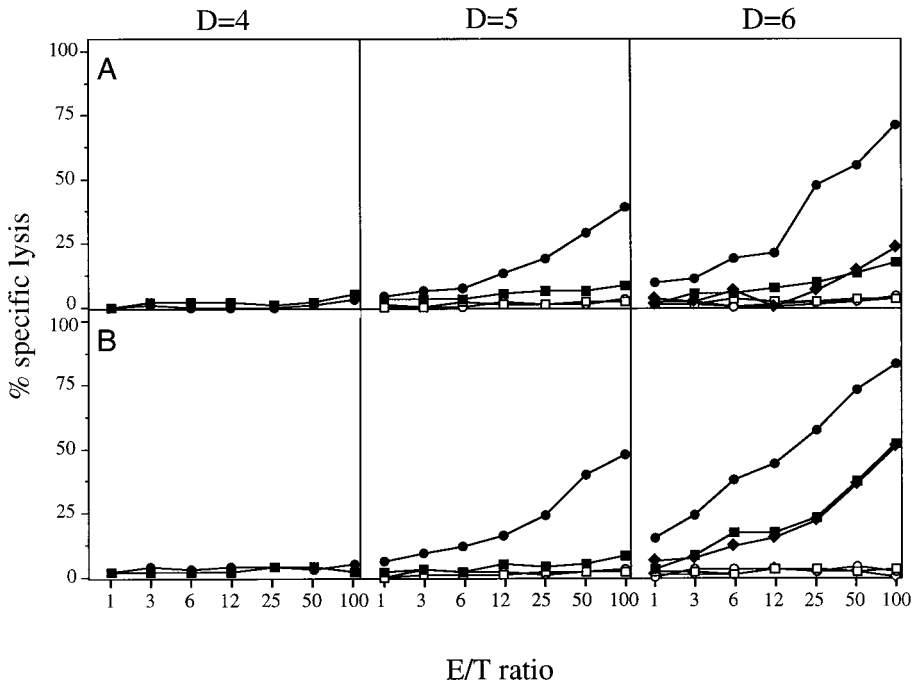

FIG. 6. Effects of increased virus titers on the kinetics and magnitude of virus-specific primary CTL responses. Groups of five PBS-treated (■), PBS-liposome-treated (◆), or Cl_2MDP-liposome-treated (●) mice were inoculated i.n. with $10^{4.5}$ (A) or $10^{6.5}$ (B) PFU of Mem71, and the virus-specific CTL activity in lung cell suspensions was assayed in a ^{51}Cr release assay on days (D) 4, 5, and 6 after infection. Open symbols, specific lysis of noninfected target cells; closed symbols, lysis of Mem71-infected target cells.

ics of the virus-specific CTL response. PBS-treated, PBS-liposome-treated, or Cl_2MDP-liposome-treated mice were inoculated with either $10^{4.5}$ PFU or $10^{6.5}$ PFU of Mem71; on days 4, 5, and 6 after infection, virus-specific CTL activity was assayed in lung cell suspensions. The experiment was designed in such a way that all ^{51}Cr release assays with the different groups of mice were performed on the same day. In this way, variations in the detected CTL activity due to variability between the ^{51}Cr release assays could be excluded and the results for each group as presented in Fig. 6 can be compared with each other. Figure 6A shows the primary CTL response over time in mice infected with $10^{4.5}$ PFU of Mem71. Infection of mice with 100-fold more virus (i.e., $10^{6.5}$ PFU) resulted in enhanced virus-specific CTL responses (Fig. 6B) in both PBS-treated and Cl_2MDP-liposome treated mice as well as in PBS-liposome-treated mice, but CTL activity in lung cell suspensions from AM-depleted mice was still higher than that in cell suspensions from normal mice. Importantly, immunization with a higher dose of Mem71 did not result in the same shift in kinetics as was seen after depletion of AM. On day 5 after infection with $10^{6.5}$ PFU, primary CTL were detectable in lung cell suspensions from Cl_2MDP-liposome-treated mice only. These results suggested that the change in the kinetics of the virus-specific CTL response in AM-depleted mice was not due to elevated virus titers.

Effect of AM depletion on virus-specific CTL responses in the MLN. Induction of antigen-specific immune responses against airborne antigens is supposed to occur in the lymph nodes draining the respiratory tract. Depletion of AM resulted in increased CTL responses in the lungs, and since it has been established that AM not only suppress T cells but also suppress the accessory cell activities of DC as well, the effect of AM depletion on virus-specific CTL responses in MLN cells was investigated. Groups of five normal and AM-depleted mice were infected with $10^{4.5}$ or $10^{6.5}$ PFU of Mem71; on days 4, 5, and 6 after infection, MLN cell suspensions were assayed for virus-specific CTL responses. Figure 7 shows that primary virus-specific CTL responses in MLN cells were maximal 5 days after infection. However, CTL activities were very low in normal mice, and specific lysis was detected only in AM-depleted mice. Immunization with 100-fold more virus (i.e., $10^{6.5}$ PFU) did not result in increased CTL responses on day 5, again suggesting that the elevated response in Cl_2MDP-liposome-treated mice was not due to elevated virus titers. On day 4 following Mem71 infection, only very low CTL responses were measured; these were only slightly enhanced in mice immunized with a higher dose of Mem71. Flow cytometric analysis of MLN cell suspensions showed that the percentages of $CD8^+$ and $CD4^+$ cells in MLN cells were equivalent in Cl_2MDP-liposome-treated, PBS-treated, and PBS-liposome-treated mice (results not shown), indicating that the observed increase in CTL activity was not due to the presence of an increased number of $CD8^+$ cells in the in vitro ^{51}Cr release assay.

DISCUSSION

Immune responses in a vital organ like the lung require precise control to ensure rapid clearance of pathogenic microorganisms without substantial damage to the delicate tissue-air interface. Influenza A virus replicates in type II epithelial cells lining the respiratory tract, and lysis of virus-infected cells by CTL is regarded as the main mechanism for eradication of established primary influenza A virus infections (1, 11, 34). Clearly, such a destructive immune response demands proper control. In the present study, we demonstrate a role for AM in the regulation of virus-specific CTL responses during respiratory influenza A virus infection. AM were selectively depleted from the lungs by i.n. administration of Cl_2MDP-liposomes

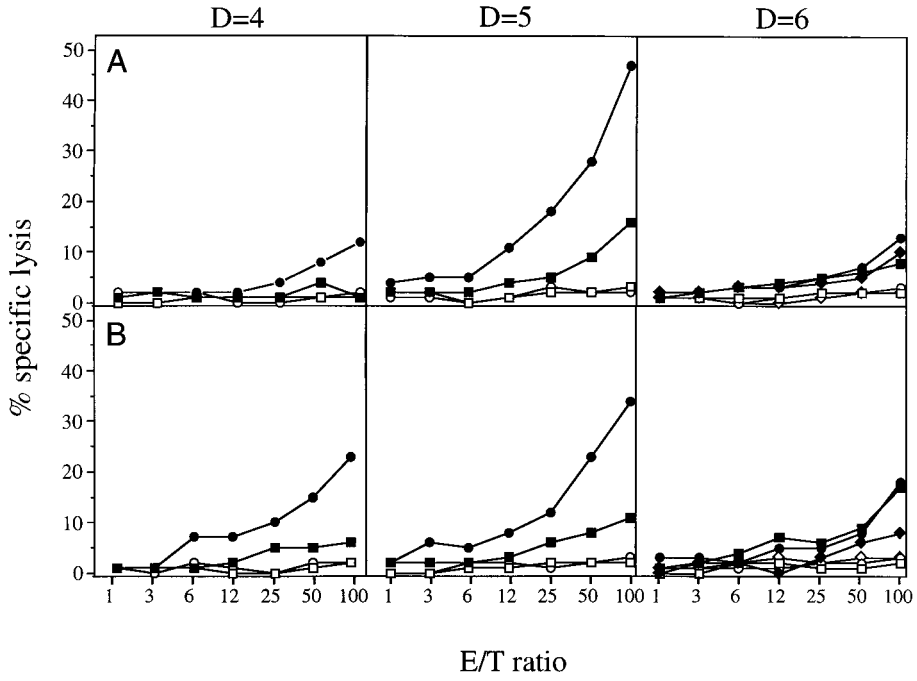

FIG. 7. AM depletion alters the regulation of virus-specific CTL responses in MLN. Groups of five PBS-treated (■), PBS-liposome-treated (◆), or Cl_2MDP-liposome-treated (●) mice were inoculated i.n. with $10^{4.5}$ (A) or $10^{6.5}$ (B) PFU of Mem71, and the virus-specific CTL activity in MLN cell suspensions was assayed in a ^{51}Cr release assay at days (D) 4, 5, and 6 after infection. Open symbols, specific lysis of noninfected target cells; closed symbols, lysis of Mem71-infected target cells.

before Mem71 infection, which changed the kinetics of the virus-specific CTL responses of MLN and lung cell suspensions and resulted in increased virus-specific CTL activities. A reduction of virus-specific CTL responses was found to be a local effect, since CTL in the spleen were not affected by AM depletion and lung cell suspensions from normal mice showed a capacity to lyse virus-infected cells which was similar to that of cell suspensions from AM-depleted mice after in vitro culturing. In addition, elimination of AM did not result in enhanced recruitment of $CD8^+$ cells or $CD4^+$ cells, since flow cytometric analysis of cell suspensions from either lungs or MLN showed similar percentages of $CD8^+$ and $CD4^+$ cells in normal mice and in AM-depleted mice.

This paper provides evidence for a role for AM in the first-line defense mechanisms against influenza virus, since elimination of AM resulted in significantly increased virus titers in the lung. AM are the first cells to encounter foreign material and pathogens because of their strategic location in the respiratory tract and are therefore regarded as the major scavenger cells of the airways (14). AM phagocytose viral particles in the lung, thereby restricting viral infiltration and reducing the viral load in the lung (12). Phagocytic uptake of influenza virus by AM may be promoted by proteins lining the mucosal surface of the lung, such as surfactant protein A, which has been shown to act as an opsonin for influenza virus uptake by AM (4). Moreover, AM from influenza virus-infected mice have been shown to have an activated phenotype (6, 16) and may provide nonspecific help by intracellular digestion of viral particles and secretion of cytokines and mediators which make surrounding cells insensitive to viral infection and which act as chemoattractants for other inflammatory cells (15, 16). In addition, AM may act as nonspecific cytotoxic cells and lyse virus-infected cells. Interestingly, both normal and AM-depleted mice had completely eradicated the viral infection within 7 days after infection, suggesting that in the absence of AM, other mechanisms help to clear the virus from the respiratory epithelium. Not only did AM-depleted mice have elevated numbers of $CD8^+$ T cells in the lung, but also the virus-specific $CD8^+$ cells had a significantly increased ability to lyse virus-infected cells. This fact, together with the fact that virus-specific cytolytic responses were detectable earlier during infection, makes it tempting to speculate that the increased CTL responses in AM-depleted mice compensated for the absence of AM and were responsible for the rapid clearance of the virus. These results therefore stress the important role that CTL may play in recovery from primary viral infections in the respiratory tract. On the other hand, enhanced CTL activities may occur at the expense of the epithelial cell layer and, in AM-depleted mice, clearance of virus may coincide with increased pathological signs.

AM have been shown to regulate pulmonary immune responses by several mechanisms both at the site of induction (the draining MLN) and at the effector site (the airways). First, AM may decrease antigen load by phagocytosis, especially of particulate antigens. Second, AM suppress the accessory cell function of DC, resulting in a reduced capacity of the latter to stimulate naive T lymphocytes (18). Finally, AM inhibit T-lymphocyte proliferation, thereby blocking the expansion of antigen-specific T cells in the lung (28, 32). The results of this study provide evidence for the use of all three mechanisms during viral respiratory infections. As discussed above, AM were responsible for the removal of viral particles, thereby reducing the viral antigen load. To determine how increased virus titers affect CTL responses in the lung, mice were inoculated with either a high ($10^{6.5}$ PFU) or a lower ($10^{4.5}$ PFU) dose of Mem71, and CTL responses were compared. The results of these experiments showed that increased virus titers induced elevated CTL responses but did not induce a shift in the kinetics of the CTL response similar to that observed in AM-depleted animals. Moreover, inoculation with a higher

virus load did not alter CTL responses in MLN. Therefore, increased CTL responses in AM-depleted mice may be due in part to increased viral antigen load.

AM are able to regulate the induction of specific T-lymphocyte responses in the draining lymph nodes by downregulation of the APC function of pulmonary DC (18). Typically, AM have been shown to migrate from the lungs to the MLN, where they reside in the paracortical area, in close proximity to DC and T lymphocytes (30). In this way, AM may exert their suppression by both downmodulating DC and inhibiting T-lymphocyte proliferation. To date, no direct evidence for a role of DC as the main APC for viral antigens has been presented. Hamilton-Easton and Eichelberger (13) reported that macrophages, DC, and B cells isolated from murine lungs and MLN after influenza virus infection contained virus and showed that macrophages and DC were able to present viral antigens to a virus-specific T-cell hybridoma in vitro. Our results show that DC are most likely the main APC for the induction of virus-specific CTL responses, since these responses were effectively induced in the absence of AM. However, it is recognized that although the accessory cell function of IM may not be as potent as that of DC, a role for IM as APC cannot be excluded, since these cells are not depleted by i.n. treatment with Cl_2MDP-liposomes.

Analysis of the results from the cytotoxicity assays showed that virus-specific CTL activity of lung cells derived from AM-depleted mice was different from the cytolytic activity of lung cells obtained from normal mice; the CTL from AM-depleted mice showed an increased ability to lyse virus-infected cells on a per-$CD8^+$-cell basis. This increased cytolytic activity may have been the result of either increased activation in the MLN or induction of a larger number of virus-specific cells, and both may have resulted from alterations in the priming of naive T lymphocytes in the MLN. Although analysis of virus-specific precursor CTL (CTLp) frequencies in lung and MLN cell suspensions by limiting-dilution assays would provide the best insight into this matter, we attempted to resolve this question in a more indirect manner by assaying CTL activities in MLN. Primary CTL activities in MLN peaked 5 days after infection, were difficult to detect in normal mice, but were significantly increased in AM-depleted mice. A previous report failed to demonstrate primary CTL responses in MLN (3). However, since in this previous study CTL responses were often assayed more than 6 days after infection, the peak of antiviral primary CTL activity in the MLN may have passed. Our results showed that the absence of AM results in altered regulation of CTL responses in the MLN, which not only may cause substantial damage as a result of cytolysis but also may have a profound effect on the development of antiviral immunological memory as well. In the normal mouse, only a small proportion of the virus-specific CTLp migrate from the MLN to the lungs, where they become activated and mature in effector cells. The majority of the CTLp, however, remain in the MLN to found the memory CTLp pool (19, 31). In AM-depleted mice, CTLp are activated in the MLN, which decreases the survival of CTLp and may therefore result in exhaustion of the memory CTLp pool. Future experiments will focus on how AM depletion affects the induction and maintenance of influenza A virus-specific memory CTL responses.

Finally, it has been shown that AM limit clonal expansion of T lymphocytes in the lung by inducing a state of reversible anergy that results in the inhibition of proliferation (26, 27). Our results showed that upon restimulation in vitro, $CD8^+$ cells obtained from the lungs of normal mice lysed virus-infected cells with a capacity similar to that of $CD8^+$ cells derived from AM-depleted mice, indicating that the cytolytic activity of normal cells was only temporarily suppressed. Therefore, the hypothesis that, in normal mice, the expansion of CTLp is limited and results in low but sufficient virus-specific CTL activities to clear a viral infection while restricting damage to lung epithelium seems justified.

In conclusion, the results presented here provide evidence for a regulatory role for AM in the induction and activity of influenza virus-specific CTL. It was shown that AM remove a substantial part of the viral load, suppress the induction and activation of virus-specific CTL in the MLN, and inhibit the expansion of virus-specific CTL in the lungs. Questions regarding the natural activation state of the AM during influenza virus infection remain to be answered. Bilyk et al. isolated AM from influenza virus-infected lungs and demonstrated an activated phenotype (6); activation of AM may temporarily reverse the immunosuppressive phenotype of AM and allow the induction of adequate immune responses. In this regard, it is interesting that granulocyte-macrophage colony-stimulating factor, which is produced during the early stages of pulmonary influenza virus infection (15), has been shown to modulate the lymphocytostatic activity of AM and to stimulate the migration and maturation of DC (7, 8). Further experiments are required to establish how the correct microenvironment for the induction of antiviral CTL responses is created in the presence of AM and how AM regulation contributes to the memory CTL response.

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