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Characterization of Murine Lung Interstitial Macrophages in Comparison with Alveolar Macrophages In Vitro

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The present study was performed to characterize the immunologic potential of interstitial macrophages (INT) in comparison with alveolar macrophages (AL). The data showed that AL, compared with INT, have a more efficient phagocytic potential. In addition, they have a strong microbicidal activity and secrete large amounts of reactive oxygen radicals, nitric oxides, TNF, and IFN on appropriate stimulation. They also exert strong tumoricidal and parasiticidal activities. In contrast, INT are more efficient in releasing immunoregulatory cytokines such as IL-1 and IL-6. As determined by Ab staining, INT express more MHC class II molecules and are more effective in functioning as accessory cells for mitogen-stimulated lymphocyte proliferation compared with AL. Thus, AL appear to be particularly effective as nonspecific first line defense cells against infectious agents, whereas INT are equipped to cooperate with interstitial lymphocytes in inducing a specific immune reaction. *The Journal of Immunology,* 1996, 157: 3097–3104.

The lung is the major target of constant exposure to a variety of potentially harmful environmental substances, such as microbes, allergens, or toxic substances. Resident macrophages play a central role in defending the lung against these agents. The pulmonary macrophage system consists of many different populations that are found in anatomically distinct compartments, including airways and alveolar space as well as lung tissue. Most studies of lung macrophages have used alveolar macrophages (AL),[2] which are easily accessible by bronchioalveolar lavage. Not only have these macrophages been shown to serve as scavengers for foreign Ag, they also play an important role in the regulation of the pulmonary immune responses (1–3). In the normal lung, AL are supposed to be a developmental end point stage of blood monocytes (4), whereas the interstitial macrophage (INT) may represent a precursor stage of the macrophage lineage (5, 6). In contrast to AL, these INT are far more difficult to prepare, and they are therefore comparatively poorly characterized. Until Holt et al. (7) described a method for INT isolation, a suitable methodology for efficient extraction of these cells from solid lung tissue was not available. Interstitial lung macrophages are known to be phagocytic (8), to exhibit esterase activity (9), to express Fc receptors (10), and to release arachidonic acid metabolites (9) as well as some other secretory products (11, 12). However, little is known about their microbicidal and immunologic functions during in-

flammation in the lung. There is clear evidence for particle transport from inhaled Ag into the lung tissue (13, 14), so it is of great importance to know whether INT-like AL express microbicidal and cytotoxic activities. Furthermore, if these phagocytes are capable of releasing proinflammatory cytokines, their close proximity to lymphocytes and fibroblasts within the interstitium makes them obvious candidates for playing a role in immunoregulation and development of chronic lung disease.

In the present report, we compared the functional properties of AL and INT with regard to secretion of immunoregulatory cytokines and the ability to function as accessory cells for lymphocyte proliferation. Furthermore, we compared the tumoricidal and microbicidal potentials of both populations. The data presented show that AL have a high microbicidal potential, whereas INT are better equipped for immunoregulatory and accessory functions.

Material and Methods

Mice

Cytokines and LPS

Human rIL-1, human rIL-6, and murine rTNF-α were obtained from Genzyme, Boston, MA. Murine IFN-γ was kindly provided by Boehringer Ingelheim, Ingelheim, Germany. LPS from the *Escherichia coli* strain 03B4 was purchased from Sigma Chemical Co., St. Louis, MO.

Cell lines and microbes

P815, a mastocytoma induced by methylcholanthrene in DBA/2 mice, was obtained from American Type Culture Collection (ATCC), Rockville, MD, and maintained as a tissue culture in RPMI 1640 medium supplemented with 10% FCS. 7TD1-hybridoma cells were kindly provided by Prof. Edgar Schmidt, University of Mainz, Germany, and maintained in tissue culture with RPMI 1640 medium supplemented with 10% Con A-stimulated spleen cell supernatant. D10.G4.1, a T helper cell line, was obtained from ATCC and was cultivated in Iscove medium supplemented with 5% FCS, 1% glutamine (2 mM), 2% HEPES (5 mM), 2-ME (0.5 mM), and 10% Con A-stimulated spleen cell supernatant. *Leishmania donovani,* strain LRCLD 51, was kindly provided by Dr. Ebert, Hamburg, Germany.

Antibodies

Neutralizing polyclonal Ab against TNF-α and IL-1 were obtained from Genzyme, those against IL-6 were purchased from Herbert Biermann, Bad

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[2] Abbreviations used in this paper: AL, alveolar macrophages; [³H]dThd, [³H]thymidine; INT, interstitial lung macrophages; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide; RNI, reactive nitrogen intermediates; MOI, multiplicity of infection; ROI, reactive oxygen intermediates; RSV, respiratory syncytial virus.

Nauheim, Germany, and those against IFN-$\alpha\beta$ were purchased from Lee Biomolecular, San Diego, CA. M133-13A rat anti-mouse macrophage mAb was a kind gift from Prof. Kennel, National Laboratories Oak Ridge, TN. Anti-I-A^d FITC-conjugated mAb was obtained from Paesel and Lorei, Frankfurt, Germany. MOMA-1 rat anti-macrophage monoclonal was a kind gift from Prof. Kraal, University of Amsterdam, The Netherlands. BM8 rat anti-macrophage mAb was a generous gift from Dr. Burmester, Münster, Germany. Mouse and goat serum were purchased from Vector Laboratories, Burlingame, CA. Isotype control Ab and FITC-conjugated anti-rat F(ab')$_2$ fragments were obtained from Dianova, Hamburg, Germany.

Isolation of AL

AL were harvested by a modified method previous described by Kobzik et al. (15). In brief, the lung was flushed with 1 ml of ice-cold Mg$_2$- and Ca$_2$-free PBS containing 0.6 mM EDTA (PBS-EDTA). The lavage procedure was repeated 10 times. After washing the lavage cells, differential cell counts were done. Lavage fluid from untreated mice contained 98% AL. AL were >96% viable as measured by trypan blue exclusion.

Isolation of lung interstitial cells

Isolation of INT was performed according to a method modified from that of Holt (7) and Kobzik (15). After exhaustive lung lavage, the pulmonary artery was cannulated and the left atrium was opened. The lung vascular bed was perfused with ice-cold PBS-EDTA until the tissue turned white, followed by perfusion with RPMI 1640 medium containing 66 U/ml DNase Type I (Sigma) and 50 U/ml collagenase (Worthington Type IV, Copper Biomedical, Malvern, PA) (enzyme solution). The lungs were excised, the heart, trachea, and vessels were removed, and the remaining lung tissue were sliced into 0.4-mm pieces with a McIlwain tissue chopper (Surrey, Great Britain).

The lung tissue fragments were incubated in a 15-ml enzyme solution supplemented with 10% FCS for 60 min at 37°C in a gently shaking water bath. To remove cell debris, the solution was passed through a thin layer of premoistened nylon wool. The lung-digested cells were then washed twice with RPMI 1640 medium supplemented with 10% FCS and finally layered on top of a two-step Percoll gradient (20–50%). After centrifugation at 300 × g at 4°C, the interphase between the 20 and 50% Percoll layers was harvested, washed again, and resuspended in RPMI 1640 medium with 10% FCS. To further enrich the isolated cells for macrophages, cells were seeded in microtiter plates or, depending on the assay system used, in petri dishes and washed carefully after an incubation period. The remaining cells were >95% macrophages as determined by nonspecific esterase and phagocytic activities and were designated as INT in the following text.

Ab labeling and flow cytometry analysis

Cells (2 × 10^5) were suspended in 40 ml of PBS with 2% BSA and 0.2% sodium acid (FACS buffer). Cells were incubated with 5% normal mouse serum for 20 min to prevent nonspecific binding to Fc receptors. The cells were washed and further incubated with 40 ml of specific Ab solution or with isotype control Ab in the same dilution or with medium alone for 45 min. Afterward, the cells were washed three times with FACS-buffer and stained with 40 ml of FITC-conjugated goat-anti rat F(ab')$_2$ in a final dilution of 1:40 for indirect immunofluorescence. After a 45-min incubation period, cells were washed again three times and analyzed with a FACScan (Becton Dickinson, Heidelberg, Germany) for an increase in green fluorescence (FL1). All staining and washing steps were done at 4°C.

Phagocytosis assay

Saccharomyces cerevisiae, a kind gift from Prof. Auling, University of Hannover, Germany, were heat-killed and labeled with FITC (Sigma). Macrophages were seeded in polypropylene tubes, mixed at a 1:10 ratio with labeled *S. cerevisiae*, and incubated at 37°C, 5% CO$_2$ for the indicated time period. An increase in green fluorescence due to phagocytosis was measured by flow cytometry. As a control for nonspecific binding, tubes containing macrophages and *S. cerevisiae* were incubated on ice. Furthermore, nonphagocytic P815 cells together with labeled yeast particles were incubated at 37°C, 5% CO$_2$.

IL-1 assay

IL-1 activity was determined by the ability to stimulate the proliferation of IL-1-dependent D10G4.1 cells (16) as measured by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT). One unit of IL-1 was defined as the amount of IL-1 required to stimulate 50% maximal proliferation. As a positive control, human rIL-1 was used. Preincu-

bation of macrophage supernatants with neutralizing antiserum against murine IL-1 completely abolished IL-1 activity in macrophage supernatants.

IL-6 assay

IL-6 levels in supernatant of macrophage cultures were determined by a MTT reduction assay using the IL-6-dependent B cell hybridoma 7TD1 (17). One unit of IL-6 was defined as the amount of IL-6 required to stimulate 50% maximal proliferation. Human rIL-6 served as a positive control. After preincubation of macrophage supernatants with neutralizing antiserum against murine IL-6, no IL-6 activity remained in the macrophage supernatants.

TNF-α assay

Macrophage supernatant TNF-α activities were measured in a biologic assay on actinomycin-D (Sigma)-treated L929 cells as described by Franke-Ullmann et al. (18). The units given are the reciprocal values of the supernatant dilution that would cause lysis of 50% of L929 cell monolayer. As a positive control for the cytopathic effect, human rTNF-α was used. No activity was detectable after preincubation of supernatants with neutralizing Ab against murine TNF-α.

Interferon assay

IFN content of macrophage supernatants was measured using a cytopathic assay as described by Lüttig (19). In brief, serial dilutions of supernatants were added to L929 monolayers seeded in microtiter wells. After a 24-h incubation, the plates were washed, and fresh media containing appropriate concentrations of vescular stomatitis virus were added. After a 24-h incubation, L929 cells were evaluated microscopically for cytopathic effects. IFN units were defined as the reciprocal values of the supernatant dilution that would prevent virus-induced lysis in 50% of L929 cell monolayer. Protection was completely inhibited by preincubation of supernatants with neutralizing Ab against IFN-$\alpha\beta$.

Assessment of accessory cell function

Determination of accessory functions of macrophages was performed according to a modification of the method described by Kaltreider (20). In brief, nonadherent spleen cells were prepared by nylon wool column as described by Baccarini (21). Recovery averaged 40% of applied cells. Remaining mononuclear phagocytes were <1% as determined by phagocytosis assay and nonspecific esterase staining. These fractionated spleen cells were seeded with 2 × 10^5 cells per well in microtiter plates, macrophages were added in different proportions (0–8 × 10^3 cells/well), and lymphocyte proliferation was stimulated by 2 μg/ml Con A. After 72 days of incubation, [^{3}H]thymidine ([^{3}H]dThd) was added, and blastogenesis was determined by the incorporation of [^{3}H]dThd into replicating cells. After cell labeling for 6 h, the cells were harvested by suction filtration (Skatron, Liev, Norway), and the radioactivity bound to fiberglass filter paper was finally determined by beta scintillation counting (Beckman LS 7500, Beckman Instruments, Fullerton, CA). Background radioactivity obtained from spleen cells cultured without Con A in the presence or absence of macrophages was subtracted from each probe. The Con A-induced proliferation was determined according to the formula:

$$\% \text{ control cpm} = \frac{\text{cpm fractionated spleen cells} + \text{macrophages}}{\text{cpm unfractionated spleen cell}} \times 100.$$

Unfractionated spleen cells still containing spleen macrophages were used for 100% accessory function.

Measurement of lucigenin-dependent chemiluminescence

The chemiluminescence assay has been previously described by Lüttig (19). In brief, 1 × 10^5 freshly isolated macrophages per vial were cultured in 10 mM HEPES-buffered RPMI 1640 medium supplemented with 10% FCS and incubated for 24 h. The adherent macrophages were washed gently, and fresh medium was placed into the vials. Shortly before running the assay, 0.01 ml of lucigenin (Sigma) at a concentration of 12 mM was added. When background activity, as monitored in a 6-channel Berthold Biolumat (Berthold, Wildbad, Germany), reached constant values, 10 ml of zymosan (Sigma) was added. Generation of reactive oxygen intermediates resulting in chemiluminescence was measured for a period of 30 min. Software for computerized calculation of peak activities and integrals was supplied by Berthold.

Determination of RNI

Macrophage supernatants were assayed for RNI (NO$_2^-$) by a modification of the method previously described by Ding (22). In brief, 50 ml of serial

Table I. *Influence of different conditions used for lung tissue digestion on the number of lung cells[a]*

| Time (h) | Temperature (°C) | Enzyme Concentration | | Cell Counts/Lung ($\times 10^6$) |
		Collagenase (U/ml)	DNase (U/ml)	
4	20	150	198	16.9 ± 2.0
4	20	50	66	14.9 ± 1.7
2	37	150	198	14.8 ± 2.1
2	37	50	66	12.9 ± 1.9
1	37	150	198	13.3 ± 1.5
1	37	50	66	12.0 ± 1.6
2	37	0	66	3.2 ± 1.6
2	37	0	0	1.6 ± 0.8

[a] Lung tissue was minced and incubated for the indicated time using different enzyme concentrations. Total cell counts were done after digestion and removal of cell debris by centrifugation.

dilutions of macrophage supernatant was mixed with an equal volume of 1% w/v sulfanilic acid (in 4 N HCl), and 10 ml of concentrated HCl was added and further incubated at 37°C for 10 min. After the addition of 50 ml 1% w/v *N*-(-1-naphtyl)-ethylenediamine (in methanol), absorbance was measured at 550 nm in an ELISA reader (MR 66 micro-ELISA-autoreader, Dynatech, Denkendorf, Germany). Concentrations were determined using sodium nitrite (1 mM) as a standard.

^{51}Cr release assay for cytotoxicity against tumor cells

The ^{51}Cr release assay has been described previously (23). In brief, effector cells in triplicate microtiter wells were incubated with 5×10^3 ^{51}Cr-labeled (200 mCi) P815 tumor cells at an effector to target ratio of 10:1 at 37°C, 5% CO_2 for 18 h. Aliquots of the supernatant were collected, and their radioactivity measured in a gamma counter ($cpm_{experimental}$). The percentage of specific lysis was then calculated according to the formula:

$$\% \text{ specific lysis} = \frac{cpm_{experimental} - cpm_{spontaneous}}{cpm_{total}} \times 100.$$

The spontaneous release ($cpm_{spontaneous}$) was determined by incubation of tumor cells without effector cells and never exceeded 25% of the total radioactivity (cpm_{total}).

Leishmanicidal assay for cytotoxicity against microorganisms

The leishmanicidal assay method has been previously described by Hockertz (24). [^{3}H]dThd (70 mCi, 25°C for 24 h)-labeled *L. donovani* promastigotes were added to macrophages with an effector to target ratio of 1:5. After an incubation period of 18 h at 37°C in 5% CO_2, the plates were centrifuged, and half of the supernatants were collected for reading the radioactivity in a beta scintillation counter (Beckman LS 7500, Beckman). The percentage of specific lysis was calculated according to the formula

mentioned above. The total release of [^{3}H]dThd was determined by lysis of labeled *L. donovani* promastigotes using 0.01% SDS for 24 h.

Statistical analysis

Each experiment was done in triplicate. SEM were calculated for in vitro assays, and differences were analyzed by means of Student's t test.

Results

Isolation of INT

INT were isolated using different enzymatic conditions for digestion, leading to different numbers of isolated lung cells. Some examples of the chosen conditions are listed in Table I. To address the question of whether the digestion procedure influences the functionality of the macrophages, we treated isolated AL with the same conditions used for isolation of INT and measured specific functional parameters afterward. Depending on the enzyme concentration used for digestion, we observed a reduction in AL viability as well as a change in macrophage function.

The influence of varying enzyme concentrations on macrophage viability and on macrophage function during a digestion period of 1 h at 37°C is presented in Table II. Together, isolation of INT with a lower enzyme concentration (50 U/ml collagenase, 66 U/ml DNase) combined with a shorter digestion period (1 h, 37°C) resulted in a reliable number of cells with only minimal influence on macrophage viability as well as on macrophage function. Therefore, these conditions were used further to isolate INT for functional analysis.

Surface marker of pulmonary macrophages

To characterize surface markers of pulmonary macrophages, we labeled AL and INT with various Ab for FACS analysis, as summarized in Table III.

First, we determined the localization of the INT population within the "dot-plot" display of the FACS measurement. Therefore, we performed a phagocytosis assay with isolated nonadherent lung interstitial cells as well as with adherence-enriched INT for flow cytometry measurement. Based on this information, we were able to define a specific gate for INT within the dot-plot display, which we used for analysis of the Ab-labeled lung cells.

As shown in Table III, the INT as well as AL were almost 100% positive for the lung macrophage marker M133-13A (25) and were strongly labeled by this Ab. The macrophage marker F4/80 was also present on lung macrophages although the staining was not as intensive as with the M133-13A (data not shown). Both populations contained a minor subpopulation of cells expressing Mac-1 Ag. In contrast to AL, the macrophage maturation marker BM8 (26) was completely absent from INT, whereas MOMA-1 (27)

Table II. *Influence of varying enzyme concentration on macrophage viability and certain functional parameters after an incubation period of 60 min at 37°C*

| Enzyme Concentration Used for Digestion | | Parameters Investigated | | | | | |
Collagenase (U/ml)	DNase (U/ml)	Viable cells (%)[a]	Phagocytotic cells (%)[b]	TNF-α secretion (U/ml)[c]	M133 13-A-positive cells (%)[d]	ROI release ($\times 10^5$ cpm)[e]	RNI release (μM)[f]
150	198	82 ± 6.1	42 ± 5.2	460 ± 65	89 ± 4	158 ± 13	8.5 ± 1.5
100	132	95 ± 3.0	48 ± 6.9	410 ± 30	93 ± 4	157 ± 9	8.6 ± 1.4
50	66	96 ± 2.8	55 ± 4.8	460 ± 50	97 ± 2	151 ± 10	8.4 ± 1.2
0	0	98 ± 1.0	60 ± 5.4	450 ± 40	98 ± 2	142 ± 11	8.3 ± 1.3

[a] Determined by trypan blue exclusion.
[b] Determined by phagocytosis assay with FITC-labeled *S. cerevisiae* (30 min, 37°C).
[c] TNF-α secretion of adherent macrophages was measured after 18-h incubation with LPS; no TNF was detectable without previous stimulation.
[d] Determined by Ab labeling and FACS analysis.
[e] Spontaneous ROI release measured as chemiluminescence.
[f] Spontaneous RNI release.

Table III.   *Determination of Ag expression on AL and INT suspension, done by Ab labeling and FACS analysis*

	Abs Used:	Ab Labeling of AL	Ab Labeling of INT
Abs against mature macrophages	BM 8	+	−
	M133 13-A	++	++
Abs against macrophages in certain differentiation or activation stages	MOMA 1	−	+/−
	Mac-1	+/−	+/−
	MHC 2	+/−	+
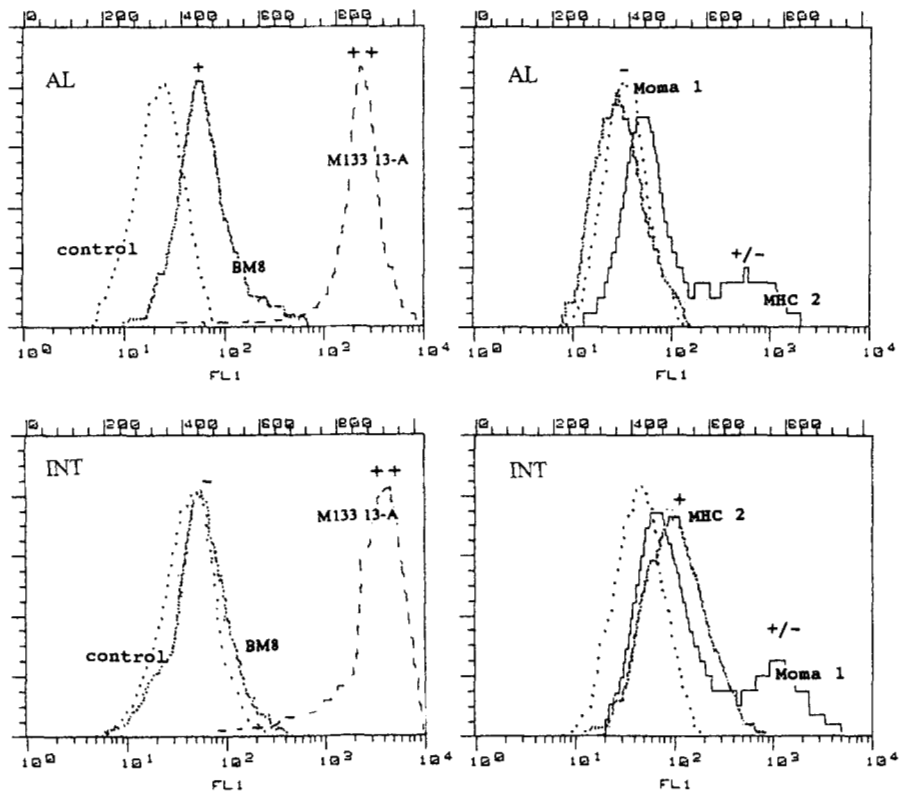
[a] Classification of labeling intensity: −, no antigen expression; +, dim antigen expression; ++, bright antigen expression; +/−, only a subpopulation was labeled.

Ag-positive cells, which are supposed to play a role in Ag presentation, were only present on the INT macrophage population. Of interest, the INT population contained more MHC class II Ag-expressing cells than the AL population.

Phagocytosis rates of lung macrophages

Both lung macrophage populations were tested for their phagocytic activity, as described in *Materials and Methods*. AL and INT were incubated in vitro with FITC-labeled *S. cerevisiae* and allowed to phagocytose for 3 h at 37°C. Control cells were kept at 4°C during the phagocytosis assay. Furthermore, the nonphagocytic cell line P 815 was used to estimate nonspecific binding. As shown in Figure 1, AL clearly have a higher phagocytic capacity at all time points tested.

Secretion of cytokines involved in defense against microorganisms or tumor cells

Macrophages are known to produce many effector molecules facilitating the killing of potentially harmful organisms and tumor cells. ROI, RNI, TNF, and type I IFN belong to the most effective mediators of such defense mechanisms, so we examined the release of these four substances. The spontaneous release of these

mediators was quite similar for INT and AL, with the only exception being ROI production where AL already had a considerable spontaneous production, as shown in Figures 2 and 3. In contrast, preactivation with LPS resulted in an increase of TNF, ROI, and RNI release in both macrophage populations, but AL exhibited a much higher potential in comparison with INT, as shown in Figure 2. To assess stimulated release of IFN, we used exposure to a pulmonary virus (respiratory syncytial virus) instead of LPS for macrophage activation, yet AL displayed a much higher release of IFN type 1 after virus infection than INT (Fig. 4).
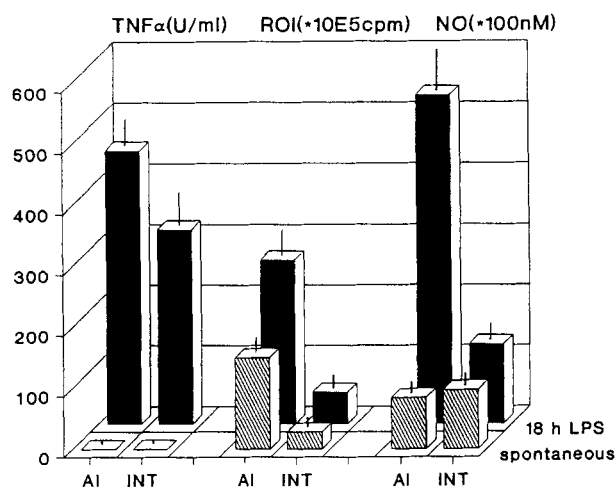
More complex functions are necessary for the lysis of tumor cells or parasites. To verify whether the differences observed for the release of cytotoxic effector molecules were also true for the destruction of tumor cells and parasites, LPS-preactivated alveolar and INT were coincubated with radioactive, prelabeled P815 mastocytoma cells or *L. donovani* promastigotes. As presented in Figure 4, *L. donovani* as well as P815 tumor cells were killed by AL with a significantly higher efficiency than by INT.

Susceptibility to viral infection

To know whether the two lung macrophage populations differed in their susceptibility for an infective challenge, respiratory syncytial
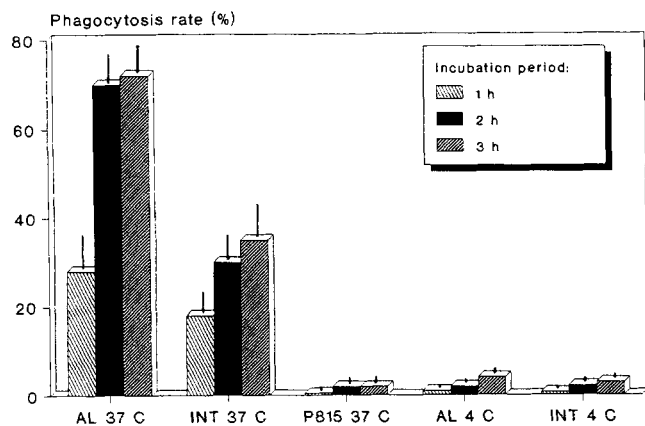
FIGURE 1. Phagocytotic potential of AL and INT were determined by addition of FITC-labeled *S. cerevisiae* to macrophage populations in fluid culture. Uptake of *S. cerevisiae* was measured at different time points by flow cytometry. Macrophages incubated on ice as well as P815 tumor cells were used for evaluation of nonspecific adherence of *S. cerevisiae* to cells.
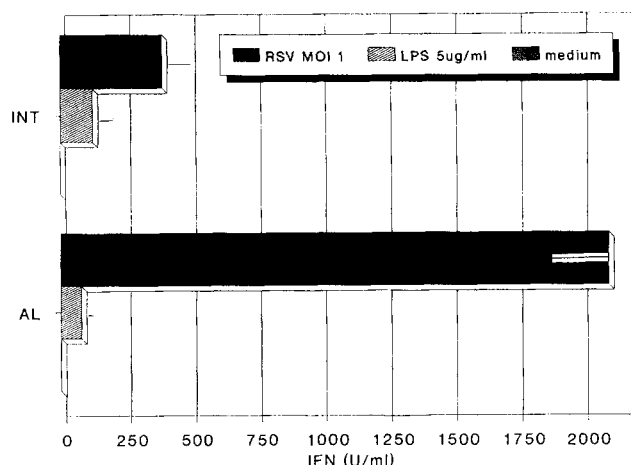

FIGURE 3. Supernatants of AL and INT were measured for their IFN concentrations after incubation for 18 h with medium alone, with 5 mg/ml LPS, or after infection with RSV (1 MOI). The IFN content of the supernatants was determined using a L929 bioassay.

FIGURE 2. Pulmonary macrophages (5×10^4) were incubated for 18 h in medium or in medium containing LPS (2, 5 mg/ml). The amount of TNF and RNI released during this incubation period was determined in L929 assay and nitrite assay, respectively, as outlined in *Material and Methods*. Zymosan-stimulated production of ROI was measured for a period of 30 min.

FIGURE 4. Cytotoxicity of AL and INT was measured as lytic activity against protozoa and tumor cells. Macrophages (5×10^5) were incubated for 18 h either with radiolabeled *L. donovani* or with P815 tumor cells. For macrophage activation, LPS (100 ng/ml) together with IFN-γ (100 U/ml) was added before the assay.

virus was chosen as infective agent. As shown in Figure 5, infected INT contained more virus than infected AL when tested in a plaque-forming unit assay on Hep-2 cells. Also, using an infection center assay, it was obvious that AL released less virus than their interstitial counterparts.
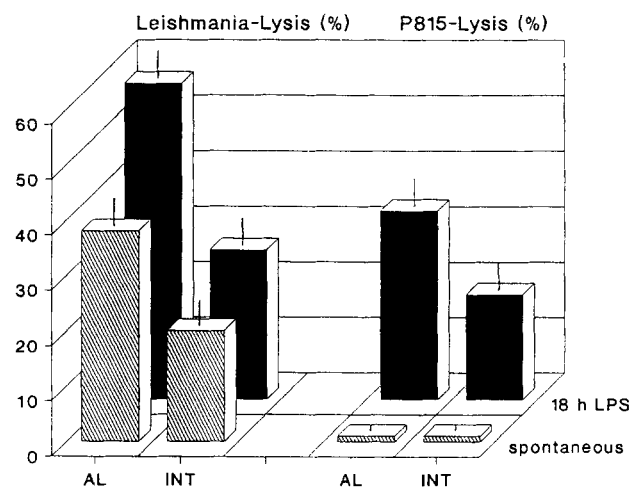
Secretion of cytokines involved in immunoregulation and accessory function

Macrophages also take part in the induction of a specific immune response through secretion of cytokines such as IL-1 and IL-6, which are especially important for the accessory cell function of macrophages. Under unstimulated culture conditions, AL and INT secreted equivalent amounts of IL-1 and IL-6 (Fig. 6). In contrast to the differences observed for cytotoxic functions, LPS-preactivated INT secreted much more IL-1 and IL-6 compared with AL.

Furthermore, both lung macrophage populations were examined for their ability to function as accessory cells in mitogen-induced

blastogenesis. Graded percentages of AL or INT were added to macrophage-depleted spleen cells, and the proliferative response in the presence of Con A was measured 72 h later. Proliferation was expressed as the percentage of the mitogen-induced response of unfractionated spleen cells, which is defined as 100% control proliferation. As shown in Figure 7, AL were clearly less effective as interstitial cells in functioning as accessory cells.
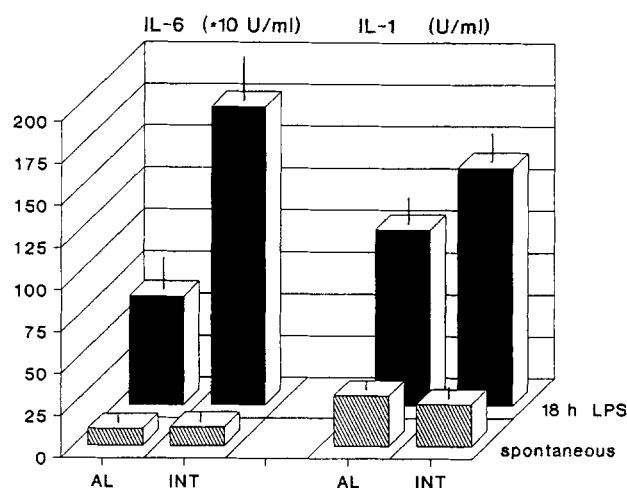
To summarize, it is shown that AL have greater cytotoxic and microbicidal potentials compared with the INT population. The INT, however, have a higher potential in the secretion of IL-1 and IL-6 along with a better accessory cell function.

Discussion

The pulmonary macrophage population consists of several subpopulations according to their different anatomical sites. The two largest populations are the AL, which are readily recovered by bronchioalveolar lavage, and the INT, which are embedded in the lung tissue and may only be removed by more rigid techniques like
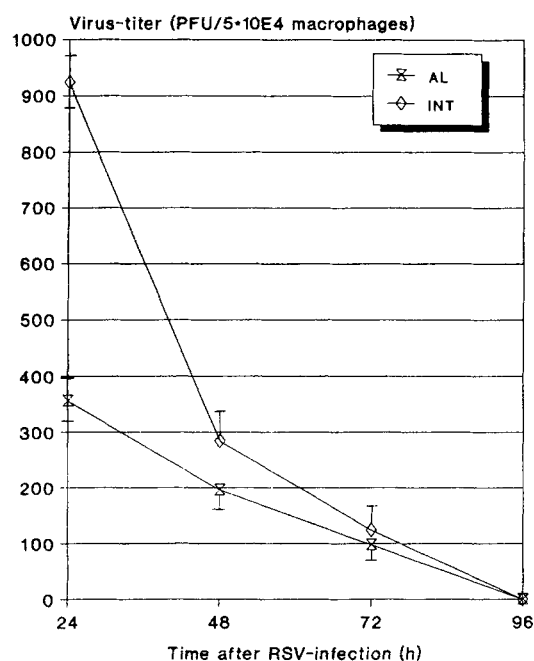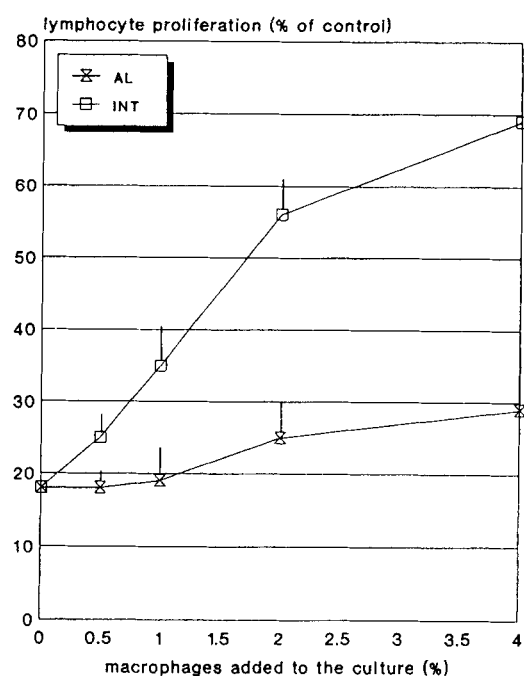
FIGURE 5. AL and INT were infected with RSV at a MOI of 1. Virus load of the macrophages was determined at the time points indicated.

FIGURE 7. Con A-induced proliferation of nonadherent spleen cells was determined after incubation with different percentages of AL or INT. Proliferation of unfractionated spleen cells was defined as 100% proliferation.

FIGURE 6. Secretion of IL-1 and IL-6 from macrophages was measured after 18 h of incubation time. Pulmonary macrophages were incubated either in medium alone or in medium containing LPS (2, 5 mg/ml). Supernatants were assayed for their cytokine content.

enzymatic digestion. AL are located at the first line of nonspecific defense in the lower respiratory tract and may therefore represent the major effector cell in antimicrobial defense. The location of INT in close proximity to the respiratory epithelium suggests a similarly important function in local immune defense, as well as a potential key role in immunoregulation.

Here we used a method previously described by Holt and Kobzik (7, 15) to isolate pulmonary tissue macrophages. The INT of mice are smaller cells and are less granulated than AL; it is also the case in other species (11, 15). In contrast to the experiments done with hamsters and rats (7, 15), we observed functional changes of the isolated mouse macrophages by using the same enzyme concentration as recommended for digestion of lung tissue in those species. However, after reducing the enzyme concentrations, there were virtually only negligible changes in macrophage

functions due to the digestion procedure. For functional investigations, it is of great importance to use macrophage populations of high purity. Using enzymatic digestion, density-gradient centrifugation, and a final adherence step, we obtained a cell population of almost 100% phagocytic cells exhibiting a high activity of nonspecific esterase. Although we cannot entirely rule out the possibility of contaminating monocytes in the preparation of INT, it was shown that lung perfusion before lavage effectively removes blood monocytes from the lung vascular bed (28).

Taking into consideration the investigation of Lehnert et al. (29), there might be a contamination of AL remaining in the alveolar space despite intensive lung lavage, which might have influenced the functional parameters of the INT. Therefore, the differences in functional parameters between both lung macrophage populations observed might be even more pronounced.

To obtain insight into the cytotoxic potential of the tissue macrophage population, a comparative study of AL and INT was conducted. The release of reactive oxygen, nitrogen intermediates, and type 1 IFN played an important role in mediating macrophage antimicrobial defense (30, 31). AL were shown to be highly effective in ROI and RNI production, especially when preactivated with LPS in vitro, whereas INT exhibited only a small increase on stimulation. The production of RNI are known to contribute to the microbicidal function of macrophages (32), so the low potential of INT to secrete RNI either spontaneously or after activation might be in part responsible for their low capability to lyse *L. donovani* in vitro, which have been shown to be particularly sensitive to RNI. Likewise, the only slight increase in ROI release after activation might further contribute to the low leishmanicidal activity of activated INT. The release of ROI is induced during phagocytosis (33), which is less effective in INT compared with AL, and that might be the reason for the reduced ROI release from INT macrophages. Anyway, according to the location of the INT population in the lung tissue, a high capability to release RNI and ROI

could be quite harmful because both mediators are known to induce tissue injuries (34, 35), whereas for AL, because of their continuous exposure to inhaled infectious agents, effective microbicidal functions are obviously an advantage. The defense mechanisms useful against bacterial microorganisms were apparently more efficient in AL as was the defense against the viral infection tested. As has been shown, AL macrophages respond to RSV infection with a much more pronounced production of type I IFN compared with INT. Accordingly, INT are more susceptible to RSV infection than AL, as verified by the higher virus load and release of virus by INT.

In contrast to other investigations in which human pulmonary macrophages have been described as not capable of direct tumor lysis (28, 36), here we clearly demonstrate tumor cell lysis through the action of preactivated AL. Pulmonary macrophages were capable of lysing tumor cells either directly TNF-independently or through secretion of TNF-α. Both tumoricidal functions were also shown for INT, but to a lesser extent. The discrepancy between our data and previous reports might easily be explained by the utilization of different stimulation procedures for macrophage activation. Neither stimulation with macrophage activating factor (MAF) (28) nor LPS (36) alone has been shown to be sufficient for the induction of TNF-independent tumoricidal activity in pulmonary macrophages. Therefore, we used a combination of IFN-γ and LPS for induction of this cytotoxic function.

Several studies have demonstrated that AL secrete relatively little IL-1 and may therefore function poorly as accessory cells (2, 37, 38). We raised the question of whether this is also true for INT. The major characteristics of accessory cells that are required for lymphocyte stimulation are the ability to elaborate specific cytokines and the expression of MHC Ag. For optimal T cell stimulation, not only is IL-1 required (39), but secretion of IL-6 is required as well (40). Although both macrophage populations produce similar amounts of IL-1 and IL-6 spontaneously, INT were more effective in cytokine release after stimulation with LPS in vitro. In contrast to the low accessory function of AL, as has also been seen in other species, tissue macrophages showed a much higher capacity to stimulate lymphocyte proliferation. This might be due to the higher potential for release of IL-1 and IL-6 and the increased percentage of MHC Ag-expressing macrophages within the INT population in comparison with the AL population.

Both INT and AL showed high expression of M133-13A lung macrophage Ag, but exhibited a different pattern of Ag by labeling with Ab against MHC class II MOMA1 and BM8 Ag. MOMA1-positive cells were found only in the INT population. Other investigations have demonstrated that only a subpopulation of murine macrophages and no monocytes express MOMA1 Ag (41), and those positive cells are considered to play a role in Ag presentation (27). Besides the MOMA1 expression, the INT population also contained more cells expressing MHC class II Ag in comparison with AL. This is not due to the isolation procedure, because a similar result has been described for INT in lung tissue sections (42, 43). In contrast, only AL and no INT were labeled with the BM8 Ab. AL may represent a more mature macrophage stage because this Ab recognizes a maturation Ag of murine macrophages that is absent from monocytes and bone marrow macrophages (26).

There is some evidence to consider INT as somehow between the maturation stage of monocytes and AL (44, 8). This hypothesis is based on the idea that the development of macrophages occurs in different stages, characterized by certain functional properties, as has been shown by Van der Meer (45) for the development from monoblasts to bone marrow macrophages. Comparative studies between AL and monocytes have shown that AL release lower amounts of IL-1 and express almost no MHC class II Ag but have

a higher potential of TNF secretion than monocytes (43, 46, 47). Here we observed the same relationship between interstitial and AL. Therefore, our data support this hypothesis to some extent.

In summary, we compared the functional capacity of AL and INT with respect to nonspecific defense mechanisms against microorganisms and immunoregulatory functions. Our results suggest that AL are particularly effective with regard to nonspecific first line defense against aerogenic infections, whereas INT are equipped with a particularly high efficiency as immunoregulatory and accessory cells.

Acknowledgments

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