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Modulation of Mitogen-Induced Proliferation of Autologous Peripheral Blood Lymphocytes by Human Alveolar Macrophages

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Experiments were carried out to determine the effect of cocultivation of T-cell-enriched human peripheral blood lymphocytes with autologous alveolar macrophages on mitogen-induced proliferation as determined by [^{3}H]thymidine uptake. Cells obtained by fiberoptic bronchoscopy and saline bronchial lavage from 14 normal volunteers were enriched for macrophages by adherence in plastic dishes for 1 h in RPMI 1640 medium supplemented with 10% fetal calf serum. Nonadherent mononuclear cells were prepared from heparinized venous blood after Ficoll-Hypaque sedimentation by passage over nylon wool columns. T-cell-enriched populations were incubated with and without alveolar macrophages, either in the presence or absence of phytohemagglutinin. In these experiments, the number of lymphocytes was held constant (10^5 per well), while the number of alveolar macrophages was varied (0.1×10^5 to 4.0×10^5 per well). Alveolar macrophages generally tended to stimulate phytohemagglutinin-induced lymphoproliferation at lymphocyte/macrophage ratios of 10:1 but consistently and significantly suppressed proliferation at ratios which approach those usually observed in recovered human bronchial lavage fluid, namely, 1:4. The suppressive effect of alveolar macrophages was observed as early as 48 h after culture initiation, while the magnitude of suppression increased with time. Suppression did not appear to be due to alteration in lymphocyte viability, nor was it sensitive to indomethacin. These results indicate that human alveolar macrophages can modulate the in vitro proliferative response of autologous peripheral blood lymphocytes. This observation may have relevance to interactions between alveolar macrophages and bronchial lymphocytes in the human lung in vivo.

Several studies have clearly shown that peripheral blood monocytes, as well as peritoneal and splenic macrophages, can modulate a variety of lymphocyte functions, including antibody production and lymphoproliferative responses to mitogens, allogeneic cells, and soluble antigens (11, 14, 25, 28–30, 33, 35, 39). There is, however, considerable debate concerning the role of alveolar macrophages (AM) in immunological processes. In vitro studies using AM obtained from rats and dogs have shown suppressive effects, while those obtained from guinea pigs and rabbits usually manifest stimulatory or mixed effects on mitogen-induced lymphoproliferation (2, 10, 13, 15–17, 31, 32, 36). There has been a paucity of information available regarding human AM function. In the present study we have investigated the capacity of human AM to modulate the proliferative response of autologous T-cell-enriched peripheral blood lymphocytes (PBL) to the mitogen phytohemagglutinin (PHA). At low AM/PBL (1:10) ratios, a tendency toward a stimulatory effect was seen, but at ratios which approach those usually observed in bronchial lavage fluids (4:1), a consistent, statistically significant suppressive effect was observed.

MATERIALS AND METHODS

Preparation of human AM. Bronchoalveolar cells were obtained from 14 healthy volunteers, 11 male and 3 female, 18 to 28 years old, by fiberoptic bronchoscopy and saline bronchial lavage as described previously (38). Two volunteers were studied a second time, at least 4 weeks after the first lavage. Twelve volunteers were nonsmokers, one was a cigarette smoker, and one smoked marijuana. No differences were observed in the studies done in cells from smokers and nonsmokers, and the data are all presented together. The protocol for this project was approved by the Human Research Committee of our institution.

The bronchoalveolar cells were washed three times with Hanks balanced salt solution with 0.1% dextrose (HBSS; GIBCO Laboratories, Grand Island, N.Y.) and then were resuspended in RPMI 1640 medium with 2.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 1% L-glutamine, 10% heat-inactivated fetal calf serum, and 50 μg of gentamicin per ml (all from GIBCO Laboratories), hereafter referred to as supplemented medium. Viability of the bronchoalveolar cells was 75 to 85% as judged by trypan blue dye exclusion. The proportion of AM present in the bronchoalveolar cell populations ranged from 78 to 91%, as evaluated by differential cell counts with acridine orange (34), latex particle phagocytosis, and nonspecific esterase staining (21). Only cells that fulfilled these characteristics and had a diameter greater than 12.5 μm were counted as AM. Bronchoalveolar cells were added to flat-bottomed wells in microtiter plates (no. 3040, Microtest-II plates; Becton, Dickinson & Co., Oxnard, Calif.) at concentrations of 1×10^4, 1×10^5, 2×10^5, and 4×10^5 AM per well in supplemented medium. The cells were incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO_2 and then washed with supplemented medium to remove nonadherent cells.

Preparation of PBL. PBL were obtained from heparinized venous blood according to a standard procedure (3). In brief, blood was diluted with an equal volume of cold HBSS, layered over cold Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) at a blood/Ficoll-Hypaque ratio of 10:4, and centrifuged (IEC model CRU-5000) at $400 \times g$ for 40 min at 4°C. The recovered mononuclear cell suspension was washed three times in HBSS. The cells were then added to a prewashed, sterile nylon wool column and incubated for 30 min at 37°C (19). The nonadherent cells were then slowly washed from the column with approximately 40 ml of supplemented medium at a concentration of 10^6 PBL per ml. The number of nonadherent cells which exhibited diffuse esterase positivity declined from 15% before column passage to 5% after column treatment; conversely, the average proportion of lymphocytes forming spontaneous rosettes with sheep erythrocytes (20) increased from 70% before column treatment to 90% after column purification. These results indicated that when peripheral blood mononuclear cells were passed through a nylon wool column, the effluent population was selectively enriched in T cells.

Preparation of PMN. Polymorphonuclear leukocytes (PMN) were also harvested by Ficoll-Hypaque sedimentation (3). They were obtained from the leukocyte-rich layer immediately above the erythrocytes. The neutrophil preparation was washed once with HBSS, and the erythrocytes were lysed with cold 0.2% sodium chloride for 30 s. An equal volume of 1.6% saline was then added, the cells were washed once with HBSS, and the concentration was adjusted to 10^6 neutrophils per ml with supplemented medium. The proportion of neutrophils in these preparations was 90 to 95%, as assessed by acridine orange fluorescence (34). PMN (10^6/ml) were treated with 50 μg of mitomycin C per ml (Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37°C. The cells were washed four times with HBSS before adjustment to the appropriate concentration for addition to PBL cultures.

Lymphoproliferative assays. PBL (10^5 cells per well) were incubated in microtiter plates either alone or with one of four alternative concentrations of AM or PMN (1×10^4, 1×10^5, 2×10^5, and 4×10^5). Parallel cultures were set up with and without PHA (purified PHA; Burroughs Wellcome Co., Greenville, N.C.). The final volume in the wells was 0.2 ml, and the final concentration of PHA was usually 2 μg/ml. The cultures were incubated for 96 h at 37°C in a humidified atmosphere containing 5% CO_2, except where indicated. Viability of mononuclear cells in the wells in the presence and absence of AM and PHA was determined by trypan blue dye exclusion at the time of harvest.

For assessment of lymphoproliferation, 1 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass; specific activity, 6.7 Ci/mmol) was added to individual wells for the final 7 h of incubation. Cells were harvested onto glass fiber filter paper disks using a microharvester (Bellco Glass, Inc., Vineland, N.J.). The filter paper disks were put into scintillation vials and allowed to dry. Scintillation liquid [consisting of a mixture of 7 g of 2,5-diphenyloxazole (PPO) and 0.6 g of 1,4-bis-(5-phenyloxazolyl) benzene (POPOP) per liter of toluene] was then added, and the radioactivity was measured in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.; model no. LS-9000). Each determination was performed in triplicate, and the uptake of [^{3}H]thymidine was expressed as counts per minute (cpm). In general, variation among triplicate samples was less than 10 to 15%. Final results were expressed as follows: change in cpm = (cpm of cultures containing PHA − cpm of background) − (cpm of cultures without PHA − cpm of background).

In preliminary experiments carried out concurrently with [^{3}H]thymidine uptake experiments, PBL were incubated with and without PHA in the presence and absence of high (4×10^5) numbers of AM and with the addition of colchicine (1 μg/ml) 6 h before harvesting. Cytocentrifuge smears were made, and blast counts were made. In PHA-treated cultures, there was a correlation between suppression of blast counts by added AM and suppression of [^{3}H]thymidine incorporation.

Effect of indomethacin on the modulatory activity of AM in lymphoproliferation. Indomethacin, in concentrations ranging from 0.1 to 10.0 μg/ml, was added directly to wells containing AM with PHA-stimulated PBL. The cultures were allowed to incubate for 96 h, and the assay for lymphoproliferation was performed as described above. Indomethacin (Sigma Chemical Co.) was dissolved in 95% ethanol at 10 mg/ml and diluted with supplemented medium (9). A vehicle control caused no change in thymidine uptake.

Statistical analysis. Statistical analysis of the data was performed using analysis of variance with logarithmic transformation (37). The means were compared using the Duncan multiple range test, and rejection of the null hypothesis was assumed at $P = 0.05$ or less.

RESULTS

Time course of proliferation of mitogen-stimulated PBL cultured either alone or with autologous AM. The results depicted in Fig. 1 as the response of a single individual and which are

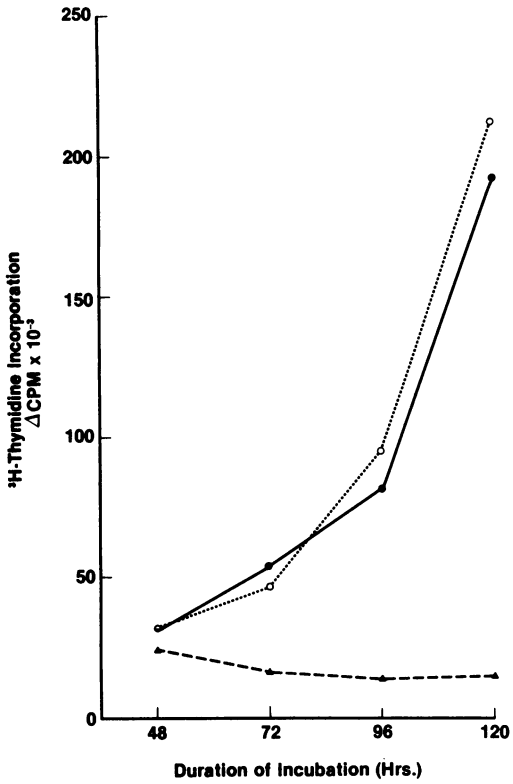

FIG. 1. Time course of [^{3}H]thymidine incorporation into PHA-stimulated (2 μg/ml, final concentration) human PBL (10^5) incubated alone (●) or with a low (1×10^4, ○) or high (4×10^5, ▲) number of autologous AM. All differences seen with 4×10^5 AM were significant; those with 1×10^4 AM were not.

representative of three separate experiments show that an increase in uptake of [^{3}H]thymidine by PHA-treated PBL was seen with time from 48 through 120 h of incubation. When AM were added at a PBL/AM ratio of 10:1, a slight increase in lymphocyte proliferation that did not reach statistical significance was seen at 96 and 120 h. On the other hand, when the PBL/AM ratio was 1:4, a ratio that might be found in the lung, significant and consistent suppression was seen at each time point from 48 through 120 h.

Effect of varying the dose of mitogen. The results presented in Table 1 demonstrate the effect of incubating PBL alone or with increasing numbers of AM and with varying doses of PHA for 96 h. It can be seen that PHA, at 1 μg/ml, stimulated maximal [^{3}H]thymidine uptake in control lymphocyte cultures. However, statistical analysis of the data showed no significant difference in the response to PHA over a dose range from 0.25 to 2.0 μg/ml. Based on these data, the upper limit of PHA (2.0 μg/ml) was selected for use in all subsequent experiments. It is clear that as the number of AM cocultured with the PBL was increased beyond 10^5, the degree of suppression of mitogenesis concomitantly increased at each dose of PHA employed. Increasing the dose of PHA above the optimum did not reverse the suppressive effect at any of the AM concentrations studied.

Effect of varying the numbers of AM cocultured with PBL. Figure 2 shows the results, expressed as the change in cpm, obtained when AM and PBL were cultured together at a fixed PHA dosage (2 μg/ml) and time interval (96 h). Low numbers of AM (10^4) produced a trend toward stimulation of lymphoproliferation in five of seven instances. This was not statistically significant. PBL/AM ratios of 1:1 produced variable results. Higher numbers of AM (2×10^5 and 4×10^5) in the culture system, however, produced consistent and significant suppression. In two individuals, repeat lavages and studies were done; a similar pattern of AM modulation of PBL proliferation was seen each time (results not shown). The viability of mononuclear cells at the end of 96 h of incubation both with and without AM was assessed and was not significantly different.

Effect of coculture of PBL with autologous PMN. To assess the cellular specificity of the changes seen in lymphocyte proliferation upon coculture with AM, PBL were cocultured with

TABLE 1. Modulatory effect of AM on autologous PBL proliferation with increasing amounts of PHA[a]

No. of AM[b]	cpm stimulated by the following doses (μg/ml) of PHA[c]:					
	0[d]	0.25	0.50	1.00	2.00	5.00
0	115	105,843	146,605	159,455	125,749	37,191
1×10^4	592	177,810	219,267	213,597	118,585	49,908
1×10^5	1,007	11,485	11,100	9,168	8,499	7,286
2×10^5	721	4,269	8,489	7,702	7,039	5,413
4×10^5	441	2,539	4,691	5,984	4,550	3,528

[a] Statistical analysis showed no significant difference in response among doses of PHA ranging from 0.25 to 2.00 μg/ml.

[b] The cpm of AM alone were always less than 500 and hence are not expressed in this and succeeding tables.

[c] cpm of 10^5 PBL in RPMI 1640 medium with PHA (with or without AM).

[d] cpm of 10^5 PBL in RPMI 1640 medium alone.

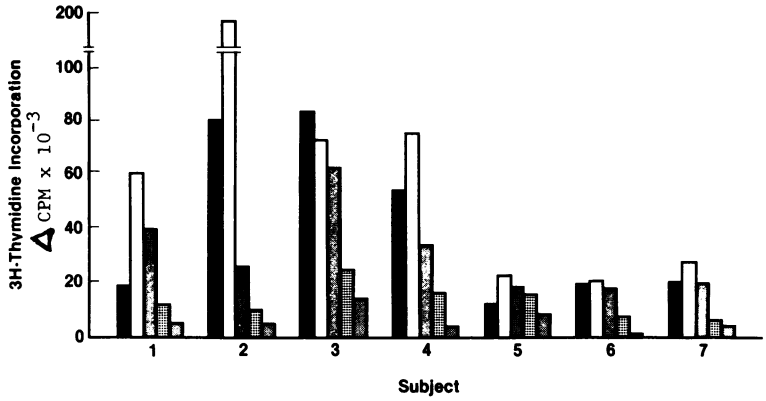

FIG. 2. Modulation of PHA-stimulated human PBL proliferation by cocultivation with autologous AM in seven experiments. The final concentration of PHA was 2 μg/ml, and all experiments were terminated at 96 h of incubation. The results obtained with cocultures of 2×10^5 AM and 4×10^5 AM reached statistical significance. Data are expressed as the change in cpm. The five bars, from left to right, in each group represent 10^5 PBL alone, PBL plus 1×10^4 AM, PBL plus 1×10^5 AM, PBL plus 2×10^5 AM, and PBL plus 4×10^5 AM.

equivalent numbers of autologous PMN. Table 2 demonstrates the expected suppression of PBL proliferation by AM at high numbers of AM, but no significant effect was observed when PBL were cocultured with autologous PMN.

TABLE 2. Results of coculture of PBL with either autologous PMN or autologous AM

Coculture conditions	cpm[a]
1×10^5 PBL alone	54,256
1×10^5 PMN alone (PMN-M)[b]	7,001 (796)[b]
2×10^5 PMN alone (PMN-M)	5,932 (458)
1×10^5 PBL plus 1×10^4 AM	112,172
1×10^5 PBL plus 1×10^4 PMN (PMN-M)	70,844 (68,392)
1×10^5 PBL plus 1×10^5 AM	38,765
1×10^5 PBL plus 1×10^5 PMN (PMN-M)	72,011 (64,770)
1×10^5 PBL plus 2×10^5 AM	18,007
1×10^5 PBL plus 2×10^5 PMN (PMN-M)	71,233 (68,236)
1×10^5 PBL plus 4×10^5 AM	10,970
1×10^5 PBL plus 4×10^5 PMN (PMN-M)	72,363 (67,795)

[a] The time of incubation was 96 h. All results reported were derived from cultures stimulated with 2 μg of PHA per ml.

[b] PMN-M were PMN that had been preincubated with mitomycin C as described in the text. Either PMN or PMN-M were used, as indicated by the parentheses. The cpm for PMN-M are given in parentheses. In each instance where PBL were incubated with PMN the result was not significantly different from that obtained with PBL alone.

Effect of indomethacin treatment on the modulation of lymphoproliferation by AM. Indomethacin, at concentrations ranging from 0.1 to 10.0 μg/ml, had no significant effect on lymphoproliferation when added to cultures containing either low numbers (10^4) or high numbers (4×10^5) of AM. Three experiments were performed, and the results of a typical experiment are shown in Table 3.

DISCUSSION

The AM plays a major role in the defense of the respiratory tract against inhaled particulate matter (18). In addition, there is increasing evidence that AM are involved, along with bronchial lymphocytes, in the local immune system operative in the lung (2, 4–6, 23, 24).

Because of difficulties in obtaining sufficient numbers of bronchial lymphocytes from normal human volunteers for detailed in vitro studies,

TABLE 3. Effect of indomethacin on human AM modulation of autologous PBL proliferation[a]

Incubation of 10^5 PBL with:	cpm
Alone	11,178
1×10^4 AM	14,014
1×10^4 AM plus indomethacin at 0.1 μg/ml	16,750
1×10^4 AM plus indomethacin at 1.0 μg/ml	6,405
1×10^4 AM plus indomethacin at 10.0 μg/ml	14,366
4×10^5 AM	3,181
4×10^5 AM plus indomethacin at 0.1 μg/ml	3,786
4×10^5 AM plus indomethacin at 1.0 μg/ml	1,909
4×10^5 AM plus indomethacin at 10.0 μg/ml	3,488

[a] The time of incubation was 96 h. All results reported were derived from cultures stimulated with 2 μg of PHA per ml.

we and others (7, 22) have elected to use autologous PBL for studies of interactions between AM and lymphocytes. The validity of using PBL to study AM function is also underscored by the recent observations of Lipscomb et al. (23), who have demonstrated that guinea pig peripheral T lymphocytes can be recruited to the lung from the circulation. It is thus likely that these recruited PBL would be able to interact with AM in the milieu of the lung. Preliminary work from our own and another laboratory has suggested that human AM are capable of suppressing mitogen-stimulated PBL proliferation in vitro (I. S. Barsoum, E. Kagan, and H. Yeager, Jr., Fed. Proc. **38**:1001, 1979; C. C. McCombs, J. P. Michalski, S. E. Brown, and R. W. Light, Am. Rev. Respir. Dis. **123**:46, 1981).

The present work demonstrates that AM from normal human volunteers can have diverse modulating effects on the lymphoproliferative response induced by PHA, depending on the proportions of AM and PBL present. With AM present at an AM/PBL ratio of 1:10, there is a tendency towards stimulation, although the trend noted is not statistically significant. This tendency was consistently observed in the cultures containing AM despite the fact that there are small numbers of contaminating monocytes present both in PHA-stimulated and control lymphocyte cultures. The fact that monocytes were not totally eliminated from the PBL population should not be of major concern since the experimental conditions reported herein more closely approximate those which would be expected to be found in the lung. It is quite clear that lung lymphocytes do not exist in the absence of AM, and even more important is the observation that monocytes are capable of migrating into the lung from the circulation (18). Therefore it is likely that AM exert their immunological effects (either positive or negative) on lymphocytes in the presence of monocytes in situ. In this study, at PBL/AM ratios of 1:4, which approach those usually found in human bronchial lavage fluid (18, 38), the predominant effect of added AM is that of suppression of lymphoproliferation.

On the basis of in vivo experiments in mice, Mackaness postulated that the function of AM is to shield the pulmonary lymphoid apparatus from antigenic assault (26). In keeping with these observations, in vitro studies using AM obtained from rats and dogs have shown suppressive effects of these phagocytes on mitogen-induced lymphoproliferative responses (2, 15). On the other hand, guinea pig AM have been reported to have enhancing effects on lymphoproliferative responses to both mitogens and antigens (10). Rabbit AM have been shown to have a complex immunoregulatory role, suppressing the specific antibody response of tracheobronchial, spleen, and popliteal lymphocytes to sheep erythrocytes, while at comparable doses, augmenting, or at high doses, suppressing, the proliferative response of lymphocyte populations to mitogens (13, 31, 32, 36). Holt applied precisely the same culture conditions in a study of the immunological function of AM from four animal species (17). He found that AM from guinea pigs and mice were stimulatory to mitogen-induced lymphoproliferation, while those from rats and rabbits were inhibitory. The highest percentage of AM used per culture was 20% (17).

Earlier studies of the effect of human AM on mitogen- or antigen-induced lymphoproliferation indicated that these cells were primarily stimulatory. Daniele et al. (7) demonstrated an enhancing effect of human AM on PHA-induced autologous PBL proliferation. In contrast to the present study, in that study, the AM were from smokers in whom striking alterations of AM morphology (12), metabolism (27), and function (38) may be present. In addition, a different number of PBL (5×10^4 per culture) and a different preparation and concentration of PHA were used than reported herein. Another report (22) demonstrated that human AM can augment antigen-induced lymphocyte proliferation and mixed leukocyte reactions. However, in that study, Laughter et al. (22) showed that AM from smokers had an impaired ability to augment these two functions. It should also be noted that AM never comprised more than 30% of the cell population in their study.

In seeking an explanation for our findings, it is possible either that we are dealing with two subpopulations of AM in our attached-cell population or that the same cells can in some way have both stimulatory and inhibitory influences on autologous lymphocytes, or perhaps both. Subpopulations of human monocytes with both variable sedimentation characteristics and variable ability to secrete prostaglandins have been described (8). Since AM are thought to be derived from blood monocytes after maturation in the interstitium of the lung (18), it is conceivable that similar functional subpopulations may exist within the AM system.

The possible mechanisms involved in the modulation of lymphoproliferation by AM require further elucidation. In other systems, macrophages have been shown to suppress lymphocyte functions through the elaboration of a variety of secretory products including prostaglandins, thymidine, arginase, and interferon (1). It would appear from the data presented in Tables 2 and 3 that the suppressive effect may be a specific one attributed to AM and that the suppression observed with higher numbers of AM

cannot be attributed to the release of prostaglandin since suppression is not reversed by the addition of indomethacin over a 100-fold concentration range. The latter finding is of interest in view of previous reports suggesting that both canine AM and human monocytes can suppress lymphoproliferation through the secretion of prostaglandin (2, 9). Alternative explanations were also considered. It is possible that AM, as effective phagocytic cells, could have removed mitogen from potential stimulation of lymphocytes. If sequestration of PHA was responsible for the suppressive effect, one would expect a shift in the AM-mitogen dose suppression when additional mitogen was added such that a higher number of AM would be required to achieve a similar level of suppression. The data presented in Table 1 argue against this as a possible explanation since there was an equivalent amount of suppression for each dose of PHA used in conjunction with a given AM concentration. Further, it is not likely that the AM-induced suppression could be attributed to the release of a cytotoxic factor from the AM since viability was equivalent in both control and AM-containing cultures at the end of the incubation period. Similar observations have been reported previously in animal model systems (13, 16). Finally, since maximal suppression was observed when 4×10^5 AM were cocultured with 10^5 PBL, one cannot overlook the possibility that the suppression could be a result of depletion of nutrients in the culture medium as a consequence of cell crowding. This would seem somewhat unlikely from the results presented in Table 2 in which it was shown that addition of an equivalent number of PMN did not result in suppression, even though these cells die after 24 to 48 h in culture. A similar conclusion was drawn in a previous study where it was demonstrated that AM and not equivalent numbers of other cell types could inhibit lymphocyte function (13).

Although the exact cellular interrelationships have yet to be defined, the present study has clearly shown that human AM can modulate the lymphoproliferative response in vitro. It is conceivable that this effect may have importance in the in vivo expression of both humoral and cell-mediated immune reactions in the human lung.

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