The Concurrent Expression of *Griffonia simplicifolia*-IB_4 Binding and Tumor Necrosis Factor-α Differs between Alveolar and Peritoneal Macrophages (43368)

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Abstract. **As a corollary to their anatomic location, alveolar macrophages (AM) have a lower threshold for generating some physiologic functions than peritoneal macrophages (PM). In this study, we examined both of these populations for their ability to bind the lectin *Griffonia simplicifolia*-IB_4 ($GSIB_4$) and to produce tumor necrosis factor (TNF)-α. The results showed that these two responses were concurrently expressed in activated macrophages, although they differed in magnitude when AM and PM were compared. Following *in vitro* incubation, AM from lipopolysaccharide-treated rats demonstrated a higher percentage of $GSIB_4$ positivity and TNF production when compared with their respective PM. Since prostaglandin E_2 can regulate the expression of some macrophage activities, experiments were conducted to determine whether this could also affect the ability of macrophages to bind the $GSIB_4$ lectin. Neither the administration of indomethacin nor exogenous prostaglandin E_2 altered the expression of this marker. Conversely, these treatments produced significant changes in TNF-α production in both alveolar and peritoneal macrophages. When the concurrent expression of $GSIB_4$ lectin binding and TNF-α production was analyzed, AM from lipopolysaccharide-treated rats demonstrated both superior $GSIB_4$ positivity and TNF-α production compared with all other macrophages examined. The results of this work show that AM and PM differ in their expression of $GSIB_4$ binding and TNF-α production. These differential responses may be important in determining the level of activity of macrophages that are participating in an immune response.** [P.S.E.B.M. 1992, Vol 199]

The concept of macrophage heterogeneity has long been known to exist both within (1–4) and between (5, 6) body compartments. Several major functional differences have been demonstrated between the lung alveolar macrophage (AM) and other mononuclear phagocytes (7), especially the peritoneal macrophage (PM) (8). *In vitro* analyses have been very useful in identifying some of these differences.

One important aspect of the functional alterations in macrophage metabolic responses lies in their ability to become activated. It is well recognized that several aspects of the activated macrophage are distinctively different from their normal quiescent counterparts (9). However, it is not obligatory that each response expressed during the process of activation be related. For example, chemotaxis and phagocytosis (10) and microbicidal and tumoricidal functions (11), as well as other physiologic activities (12), are often independently expressed in these cells. This may reflect either differential control over independent mechanisms or sequential control over the progression of more complex responses. In this regard, several of the processes involved in activation have not been completely defined. Although recent work by Paulnock and Lambert (13) has clearly provided evidence that at least some of the intermediate stages in macrophage tumoricidal activation can be determined, similar studies have not been conducted to elucidate other key interrelated functions expressed by these cells.

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In the present investigation, we examined two functional markers reputedly associated with activated macrophages. The first was the ability of these cells to bind the lectin *Griffonia simplicifolia*-IB_4 ($GSIB_4$). This moiety is specific for interacting with membrane α-D-galactose (14) and is associated with activated, rather than resident, macrophages (15). The second marker was the expression of tumor necrosis factor (TNF)-α, which is elevated in activated macrophages. This cytokine is responsible for diverse activities, ranging from mitogenic effects on diploid fibroblasts (16) to tumor cytotoxicity (17). The main purpose of this study was to determine whether there is expression of $GSIB_4$-binding ability and TNF production in macrophages. Moreover, the investigation was performed to determine whether these responses differed between AM and PM. Variations in concurrent expression could potentially be useful for fractionating macrophage populations and identifying cells in different stages of the activation process. Therefore, this study provides a basis for examining these two functions and demonstrating how they are influenced by endogenous, as well as exogenous, signals.

Materials and Methods

Macrophage Harvest/Cell Preparation. Male Fischer 344 rats (225–250 g) were intraperitoneally injected with 1.0 ml of either 0.5 mg/kg of lipopolysaccharide (LPS) (*Escherichia coli* 0127:B8; Sigma Chemical Co., St. Louis, MO) or pyrogen-free sterile phosphate-buffered saline (PBS). Three days after treatment, the animals were sacrificed via lethal intramuscular injection of 30 mg of pentabarbitol (Abbott, N. Chicago, IL). Cells were recovered via peritoneal and bronchoalveolar lavage as routinely performed in this laboratory (18–22). All cells were washed twice, enumerated using a hemocytometer, and checked for viability using trypan blue dye. Any preparations found to be less than 95% viable were discarded. Macrophages were determined by neutral red dye uptake and nonspecific esterase staining. Concentrations of the cell preparations harvested were adjusted to yield 5×10^5 macrophages/ml. Cells were then plated on sterile 12-mm glass coverslips in RPMI 1640 medium (Gibco, Grand Island, NY) + 10% fetal calf serum (Hyclone, Logen, UT), 100 units of penicillin/100 units of streptomycin, and incubated for 1 hr at 37°C at 5% CO_2 in a humidified chamber. Monolayers were then washed with five bursts of warm PBS and the nonadherent cells were removed by aspiration. Macrophages were then reincubated in RPMI 1640 containing antibiotics alone, indomethacin, or prostaglandin (PG) E_2 as indicated by individual experiments. All macrophage monolayers were examined by inverted microscopy prior to use.

Fluorescein Isothiocyanate-$GSIB_4$ Assay. This procedure was performed as described previously by Tabor *et al.* (15, 22). Briefly, macrophages were fixed on coverslips for 30 min with buffered paraformaldehyde and washed twice with warm PBS reaction buffer. Coverslips were individually transferred to fresh plates and treated with 50 μg/ml of fluorescein isothiocyanate-$GSIB_4$ lectin (Sigma). Coverslips using fluorescein isothiocyanate-*Ulex europaeus* I (UEA-I; Sigma), a nonsense lectin, were similarly prepared and used as controls for nonspecific fluorescence, which was consistently less than 2%. Following gentle mixing of the overlay solution using a pasteur pipette, plates containing coverslips were incubated at 25°C in the dark for 1.5 hr on a rocking platform (Reliable Scientific, Memphis, TN) set at 2 cycles/sec. Coverslips were then removed, washed twice in reaction buffer, and directly inverted onto a drop of Fluoromount-G (Southern Biotechnology, Birmingham, AL). Slides were analyzed using an Olympus model BH fluorescent microscope equipped to read at a wavelength of 546 nm. Cells were counted in five random areas per coverslip with a minimum of 100 cells counted per area. Each variable was prepared on triplicate coverslips per animal.

TNF Assay. Immunolocalization to determine the presence of intracellular TNF was performed using a modification of the method of Chensue *et al.* (23). Paraformaldehyde-fixed macrophages were treated with methanol and then PBS-washed. The cells were then exposed to 3% H_2O_2, washed, and incubated with normal goat serum. Subsequently, all macrophage monolayers were incubated with either 1/200 rabbit anti-murine TNF-α (Genzyme, Boston, MA) or normal rabbit serum. After PBS washing, cells were then incubated with biotinylated goat-anti rabbit immunoglobulin (Vector Laboratories, Burlingame, CA). Following three washes with PBS, the macrophages were then treated with streptavidin-peroxidase (Sigma), washed with dilute acetate, and incubated 15 min with the chromophore 3-amino-9-ethylcarbazol (Sigma). The final macrophage preparations were then rinsed and counterstained with Meyer's hematoxylin. Coverslips were inverted, mounted on glass slides in glycerin buffer-mounting media, and evaluated by light microscopy. Cells containing TNF were recognized by intracytoplasmic precipitates subsequent to antibody treatment. All cells demonstrating these characteristics were scored as positive. Five random areas per coverslip with a minimum of 100 cells per area were counted. Each variable was prepared on triplicate coverslips. The release of TNF from the cells following experimental manipulations was also examined. Supernatants were collected from macrophage cultures and analyzed using a TNF-α enzyme-linked immunosorbent assay kit (Genzyme) according to the package insert.

TNF mRNA Analysis. Total RNA was isolated from macrophages according to the method of Sambrook *et al.* (24). The monolayers were solubilized in a

solution of 10 m*M* EDTA (pH 8.0) and 0.5% sodium dodecyl sulfate. After homogenization, the plate was rinsed with an equal volume of 0.1 *M* sodium acetate (pH 5.2) and 10 m*M* EDTA (pH 8.0). The entire solution was extracted with phenol. The aqueous phase was obtained and the RNA precipitated with ethanol. The quantity and integrity of the RNA was routinely tested by absorbance at 260 nm and ethidium bromide fluorescence of RNA was electrophoresed in agarose gels. The RNA was blotted to nylon membrane and probed with either an *in vitro* transcription product (antisense TNF RNA) randomly labeled by including α-^{32}P in the transcription reaction or nick-translated TNF cDNA (Genentech, South San Francisco, CA). Membranes were hybridized for 18 hr followed by successive stringency washes and autoradiography (Kodak XAR5 x-ray film) at −80°C with intensifying screens. Multiple autoradiographic exposures were quantitated by scanning on a Molecular Dynamics laser densitometer. The total RNA levels per slot were assessed by the expression of actin mRNA. The accumulation of β-actin mRNA was determined by probing the same blot with a nick-translated actin cDNA.

Cell Column Chromatography. Cell separation by column chromatography was performed using a modification of the procedure originally described by Maddox *et al.* (14). Briefly, freshly harvested alveolar and peritoneal whole cell explants were washed three times in Ca^{2+} and Mg^{2+}-free buffer and counted. In parallel, representative samples were stained for $GSIB_4$ positivity to determine the distribution of positive macrophages prior to separation. For fractionation, whole unstained cell suspensions were applied directly to multiple $GSIB_4$-Sepharose 6MB columns prepared in advance and equilibrated with buffer at 4°C. The cells were then eluted with buffer using a flow rate of 3 ml/min. The mean percentage of cells recovered per experiment was 64% of the loading input. Effluent was monitored throughout by phase contrast microscopy. The effluent fraction contained the $GSIB_4$-negative cells. $GSIB_4$-positive cells were recovered by dismantling the columns and immersing the resin in 50 m*M* α-D-galactose (Sigma), a competitive inhibitory sugar, for 30 min with vigorous agitation (250 rpm). The $GSIB_4$-positive or -negative status of both populations was verified by immunofluorescent microscopy as performed previously by Tabor *et al.* (15, 22).

Statistical Analysis. The two-tailed Student's *t* test was used to determine whether differences existed between data sets. To control for the chance of error per experiment instead of per test, the Bonferroni method was applied to all data analysis.

Results

When alveolar macrophages and peritoneal macrophages from both normal and LPS-treated animals were analyzed, intercompartmental differences in the ability of these cells to bind the lectin $GSIB_4$ were demonstrated. In all cases, the magnitude of lectin binding was greater in AM than in PM (Fig. 1). However, the differences between normal and LPS groups within the AM population were much less ($P < 0.005$) than those differences expressed when comparisons were made within the PM population. This suggested that the ability to regulate the induction of $GSIB_4$ lectin-binding activity (GLBA) varied with the type of macrophage population analyzed (i.e., AM versus PM), even though LPS activation yields the best response in all cases. The observation that AM more easily generated this marker than PM may reflect the increased level of activity attributed to AM for some functions, despite the fact that other responses remain unchanged.

Since key macrophage functions may be blocked in the presence of PGE_2 (25), we examined the effect that prostaglandins had on macrophage GLBA. Initially, we incubated the cells in the presence of indomethacin, an inhibitor of PGE_2 synthesis, to determine whether endogenous PGE_2 production would disrupt GLBA. The results showed that neither indomethacin-treated nor control macrophages (Fig. 1A) differed in their expression of lectin binding after treatment. This response was similar regardless of whether AM or PM was examined. Since the concentration of PGE_2 produced by *in vitro* macrophages alone may not be sufficient to inhibit the production of GLBA, we exogenously added PGE_2 to the macrophage cultures and incubated them prior to assay (Fig. 1B). Under these conditions, there was no difference among the experimental groups and controls in the level of GLBA. Thus, increasing the concentration of PGE_2 or blocking its synthesis did not diminish the development of macrophage GLBA.

Since AM differ from PM in their GLBA responses, we elected to determine whether there was an association between this marker and the generation of early macrophage TNF-α production. This would be important, since both of these responses are elevated in activated macrophages. Preliminary experiments were performed to determine whether there were differences between AM and PM early TNF-α production. Using the technique of immunolocalization (Fig. 2) as developed by Chensue *et al.* (23) to detect TNF-α inside the cells as a marker, the data showed that the percentage of cells synthesizing TNF-α was greater in AM when compared with PM (Fig. 3). However, this response was unlike GLBA in that significant differences were apparent between control and LPS-derived macrophages from the lung and the peritoneum. Since this measured early intracellular TNF-α production, it suggested that perhaps the synthesis of the TNF-α transcript was less in PM as opposed to AM. Therefore, experiments were conducted to examine this hypothe-

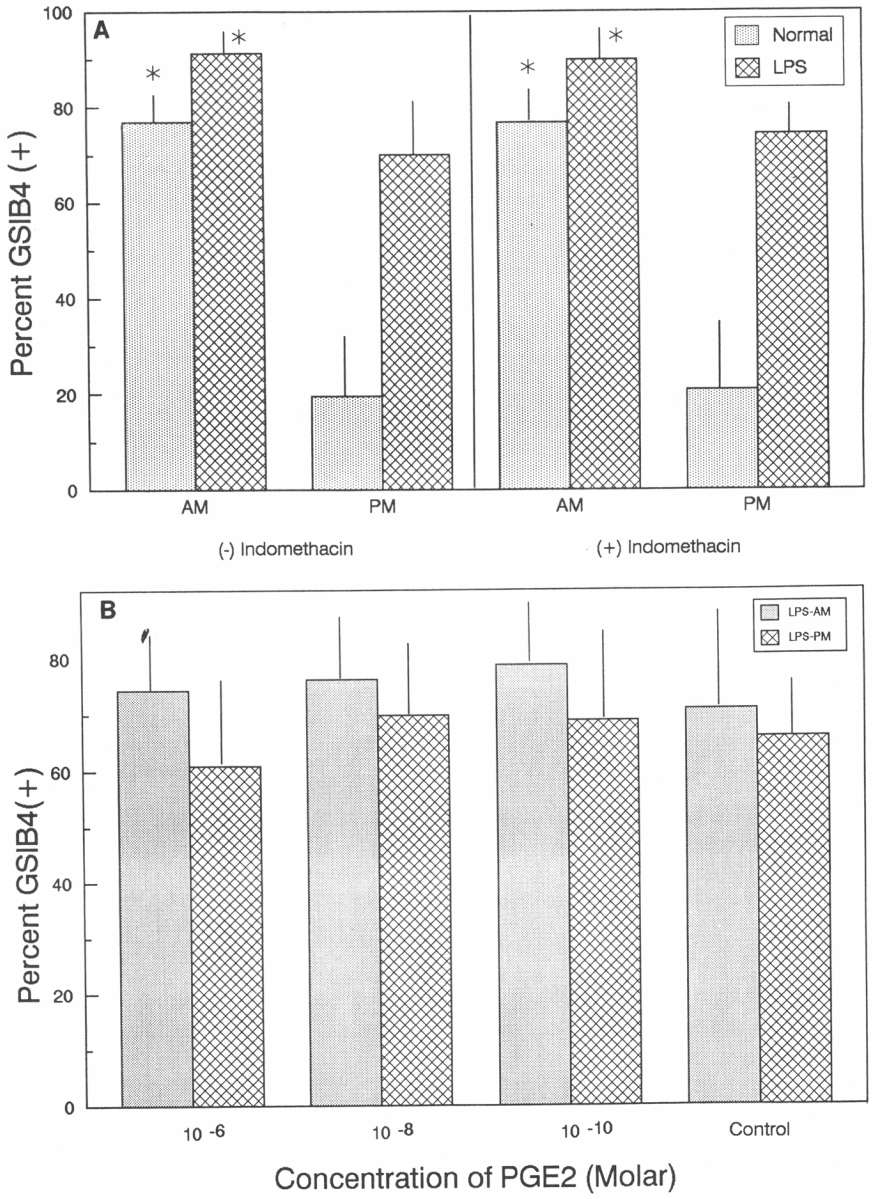

Figure 1. (A) Alveolar and peritoneal macrophages differ in their ability to bind the lectin $GSIB_4$. AM and PM from either unstimulated (normal) or LPS-stimulated animals were incubated for 4 hr with either media alone or media containing 10^{-6} *M* indomethacin. The cells were then washed and assayed for their ability to bind $GSIB_4$. Results are expressed as the mean percentage positive ± SE. *Significantly different as compared with PM, $P < 0.001$. In all cases, normal macrophages were significantly different from their pair LPS macrophages ($P < 0.005$). (B) The expression of the $GSIB_4$-lectin-binding activity is independent of direct PGE_2 regulation. AM and PM from LPS-stimulated animals were incubated for 4 hr in the presence of increasing doses of exogenous PGE_2, washed, and assayed for their ability to bind $GSIB_4$. Results are expressed as the mean percentage positive ± SE.

sis. The results of molecular studies verified that TNF-α mRNA in AM was greater than that found in PM. The data in Figure 4 show that cells from LPS-treated animals had higher TNF mRNA levels than untreated controls in both AM and PM. The relative level of increase was quantitatively similar for both cell populations (>3-fold). However, in both cases (i.e., LPS-treated and normal), AM displayed more TNF-α signal than PM. Finally, to determine whether the release of TNF-α from these two types of macrophages differed, supernatants collected from AM and PM were also analyzed (Table I). The results show that AM consistently secreted more TNF-α than did their PM counterparts. To this point then, the data showed that both the production of a transcript, the synthesis of the TNF-α protein, and its release differed between AM and PM.

When studies on AM and PM were performed in the presence of indomethacin, the production of TNF-α (Fig. 3), as well as its secretion (Table I), was elevated following inhibition of cyclooxygenase activity. These data are consistent with the results of Strieter *et al.* (26), which showed a reduction in TNF-α produced by mac-

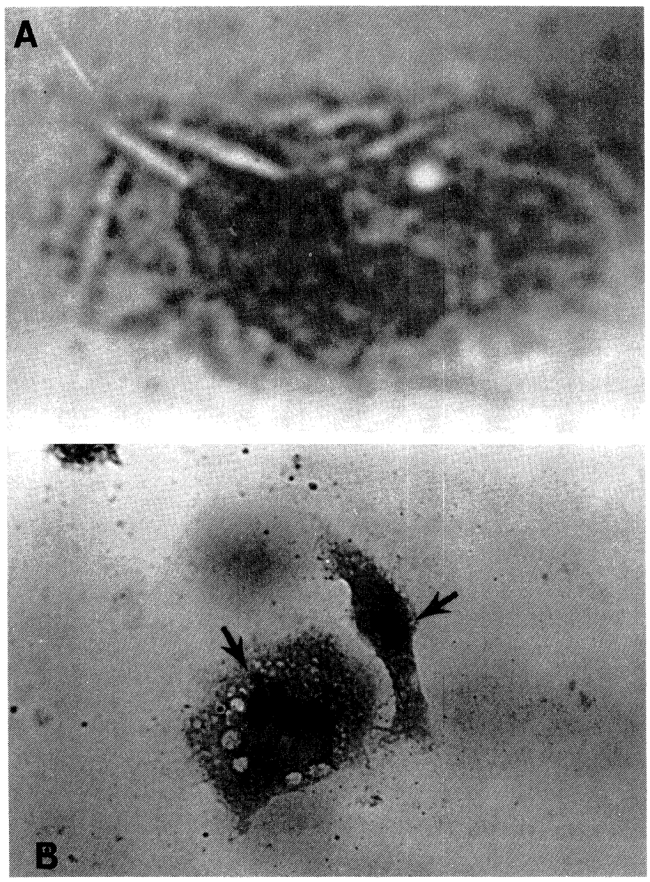

Figure 2. Immunolocalization of TNF-α within macrophages. Representative AM cultures demonstrating the presence of TNF-α in the cytoplasm (arrows).

rophages in the presence of PGE_2. However, even though inhibition appeared in both AM and PM in our work, we found that PM were more sensitive to the effects of PGE_2. In dose-response studies where PGE_2 was directly incubated with macrophages, the results demonstrated that PM TNF-α production was more markedly affected than that of AM (Fig. 5).

To this point in the investigation, each of the two markers used as indicators of activation (i.e., GLBA and TNF-α) had been independently examined in AM and PM populations. To determine whether both of these markers were concurrently expressed by macrophages, cells were first separated into $GSIB_4$-lectin binding positive ($GSIB_4(+)$) and negative ($GSIB_4(-)$) groups using cell column chromatography (Fig. 6). Freshly explanted whole cell populations were initially distributed to parallel columns, incubated, recovered, and analyzed for their marker expression. Using immunofluorescence, representative samples taken before and during each stage of chromatography were microscopically examined to determine the distribution of the cells. We found that the percentage of $GSIB_4(+)$ and $(-)$ macrophages following separation correlated with the results of the analysis performed prior to the separation processes. Therefore, the data presented were highly representative of the original populations and experimental manipulation did not alter their distributions. Macrophages from each of the two major fractions (i.e., the eluate and the retentate) were then analyzed for their ability to express TNF-α as a marker (Fig. 7) and release it (Table II). $GSIB_4(+)$ AM from the LPS-treated animals showed a significantly greater ability to express TNF-α than did their $GSIB_4(-)$ AM counterparts. A similar response was demonstrated by the PM, with the exception that their overall magnitude of TNF-α expression was significantly less than that demonstrated by the AM. Moreover, in all cases, the normal AM and PM showed significantly less TNF-α positivity than did their LPS counterparts. These data show that there is an association between GLBA and TNF production in macrophages from both of the compartments examined. However, there is significant evidence to demonstrate that expression of these activation markers is directly dependent upon the compartment of derivation.

Discussion

The AM is somewhat uniquely disposed to direct contact with the environment via the airways. This implies that at least some functions of these cells will be elevated when compared with PM. Indeed, Orens *et al.* (27) have shown that the rate of respiration of AM at rest is at least three times that of phagocytes from other body compartments. The enzymatic basis for this high rate of respiratory activity in AM is attributed to their higher levels of cytochrome oxidase as compared with PM (28). Other investigators have also shown that another important function, lysozyme secretion, is more readily produced in AM as compared with PM (29–31). However, from a teleologic point of view, it would be anticipated that some of the more potent responses should be "down-regulated" in AM in order to protect the lung from constant and severe injury. In fact, AM are much less responsive in releasing and metabolizing eicosanoids synthesized from arachidonic acid than are PM (32). From a regulatory standpoint, it is also important to note that interleukin 1 is produced in only modest levels by normal resident AM as compared with mononuclear phagocytes from other sources (33). However, this can be reversed in pathophysiologic situations that involve the stimulation of macrophages (34).

The current investigation showed that intercompartmental differences exist between the ability of AM and PM to express $GSIB_4$ binding and TNF-α production. Moreover, the results showed that both of these markers were concurrently expressed. However, as anticipated, the level of the $GSIB_4$ binding response in normal AM was innately higher than in normal PM. Although LPS stimulation enhanced this activity only

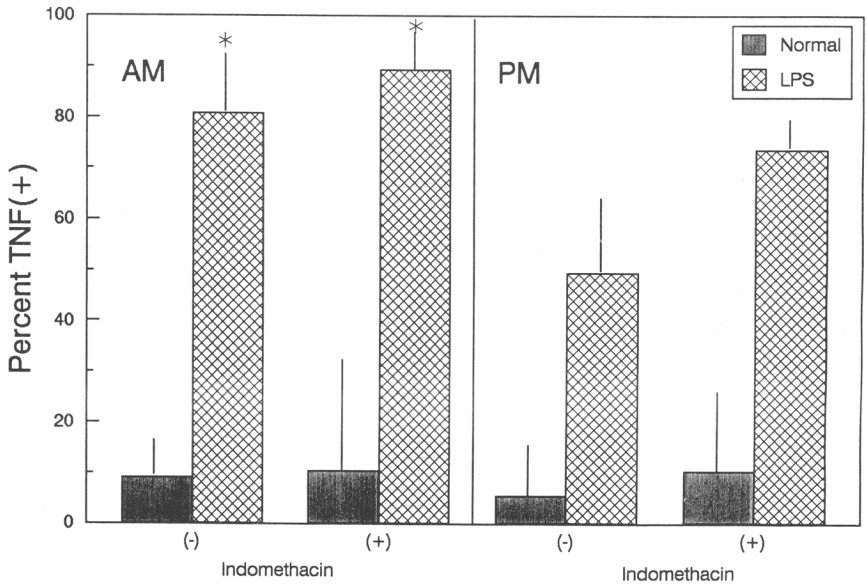

Figure 3. AM and PM production of TNF-α AM and PM from either unstimulated (normal) or LPS-stimulated animals were incubated for 4 hr with either media alone or media with 10^{-6} *M* indomethacin. The cells were washed and assayed for their ability to produce TNF-α by immunolocalization. Results are expressed as the mean percentage positive ± SE. *Significantly different as compared with PM, $P < 0.001$. In all cases, normal macrophages were significantly different from LPS macrophages ($P < 0.005$).

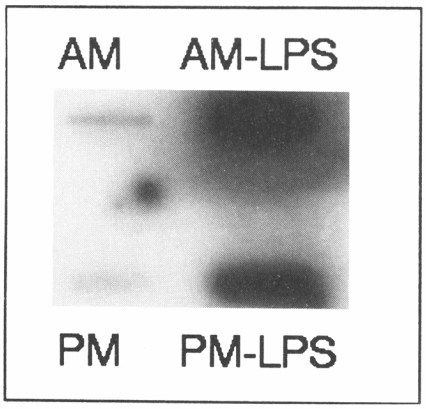

Figure 4. Detection of TNF-α mRNA in alveolar or peritoneal macrophage by slot hybridization. Total RNA was extracted from unstimulated (normal) or LPS-treated cells. RNA extracted from the cells was slotted (2 μg/slot) onto a nylon membrane, hybridized with nick-translated cDNA plasmid, and autoradiographed.

Table I. TNF-α Secretion by Alveolar and Peritoneal Macrophages[a]

	TNF-α (pg/ml)	
	(−) Indo	(+) Indo
Alveolar macrophages		
Normal	54 ± 16	80 ± 18
LPS	1623 ± 38[b]	1924 ± 26[b,c]
Peritoneal macrophages		
Normal	42 ± 15	63 ± 16
LPS	547 ± 32	951 ± 21[c]

[a] Alveolar or peritoneal macrophages from normal or LPS-treated rats were cultured in the presence (+ indo) or absence (− indo) of indomethacin. Supernatants were collected and analyzed for TNF content. The results are reported in mean pg/ml ± SE.
[b] Significantly different as compared with peritoneal macrophages, $P < 0.001$.
[c] Significantly different as compared with (−) Indo, $P < 0.001$.

modestly in AM, PM responded more markedly. Conversely, when TNF-α expression was examined, low responses from both AM and PM were significantly elevated following LPS treatment. This was at least partially anticipated based on the studies of Martinet *et al.* (35) that showed that stimulated AM generate more TNF than other mononuclear phagocytes. Collectively, the data show that the concurrent expression of these two markers is more easily demonstrated in activated as opposed to quiescent AM, while in PM, alterations in the expression of these two markers are readily apparent in both normal and activated cells. At this time, we can only speculate that in normal AM, binding of $GSIB_4$ temporarily diminishes their ability to generate TNF-α. This would not represent a unique role for lectins in that they have been described in down-regulation (36), incomplete signaling (37), and even in total antagonism (38), all of which are viable alternatives to their classic stimulatory action. Therefore, in this case, such a response may enhance the efficiency of the system by reducing the probability of continued release of mediators from normal AM. Since the PM is much less likely to become engaged in direct confrontations with microbes or foreign particulate matter on a routine basis, we suspect that their stage of readiness is significantly subdued compared with the AM. Conversely, it would be anticipated that the LPS-stimulated AM should have already achieved the threshold required for the expression of both the markers concurrently. In fact, the data show that this is the case for all the LPS-

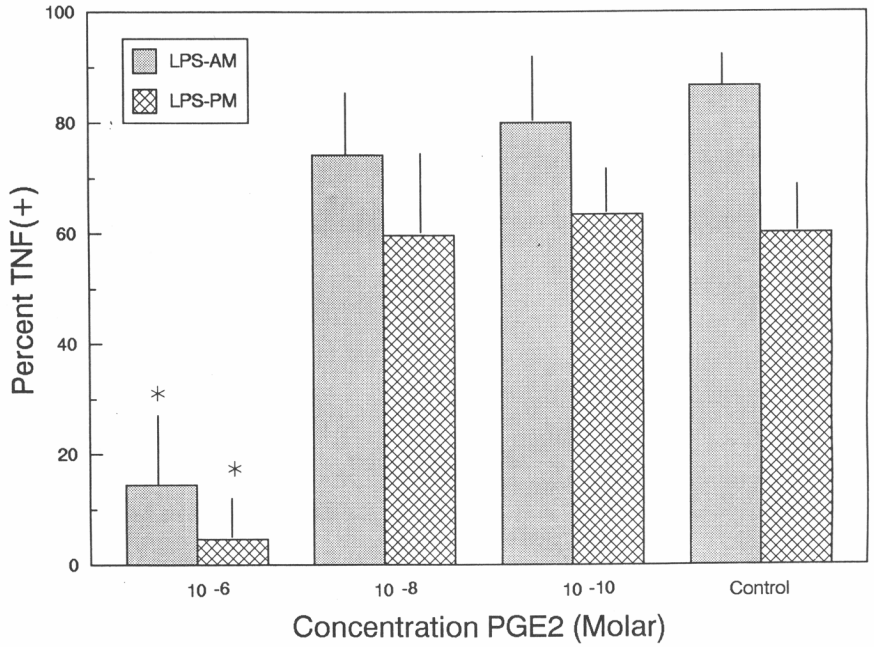

Figure 5. The production of TNF-α in AM and PM is effected by the presence of PGE_2. AM and PM from LPS-stimulated animals were incubated in the presence of increasing doses (10^{-10}, 10^{-8}, and 10^{-6} *M*) of exogenous PGE_2, washed, and assayed for their ability to express TNF-α by immunolocalization. Results are expressed as the mean percentage positive ± SE. *Significantly different as compared with control, $P < 0.001$.

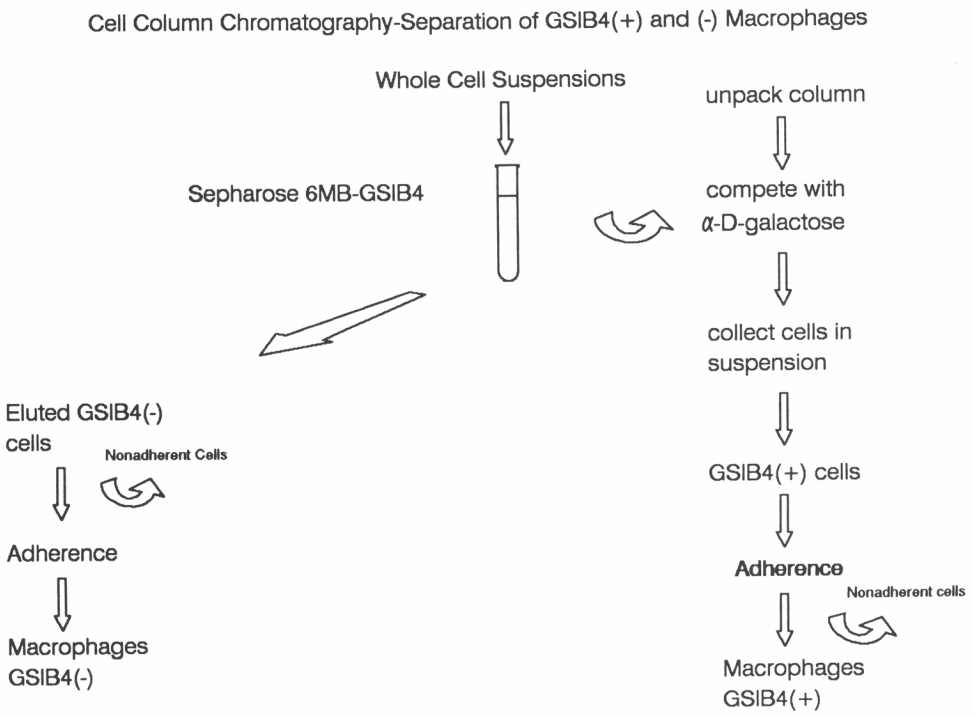

Figure 6. Fresh whole cell explants from animals were separated into $GSIB_4$(+) and (−) macrophages by Sepharose-6MB-$GSIB_4$ column fractionation (see Materials and Methods).

activated macrophages in this study. When PM were examined, however, we found that normal cells significantly differed from LPS-stimulated cells in $GSIB_4$ binding and TNF-α expression and release.

Although lectins have been used as probes for identifying some cell types (39), only a few have successfully achieved enough recognition to be consistently useful. For the most part, lectins have been used to identify abnormalities in fixed, as opposed to free, tissue cells (40–42). Although Holthofer *et al.* (43) used a general GSI-type lectin probe to identify monocytes as the cells that infiltrated human kidney during nephritis,

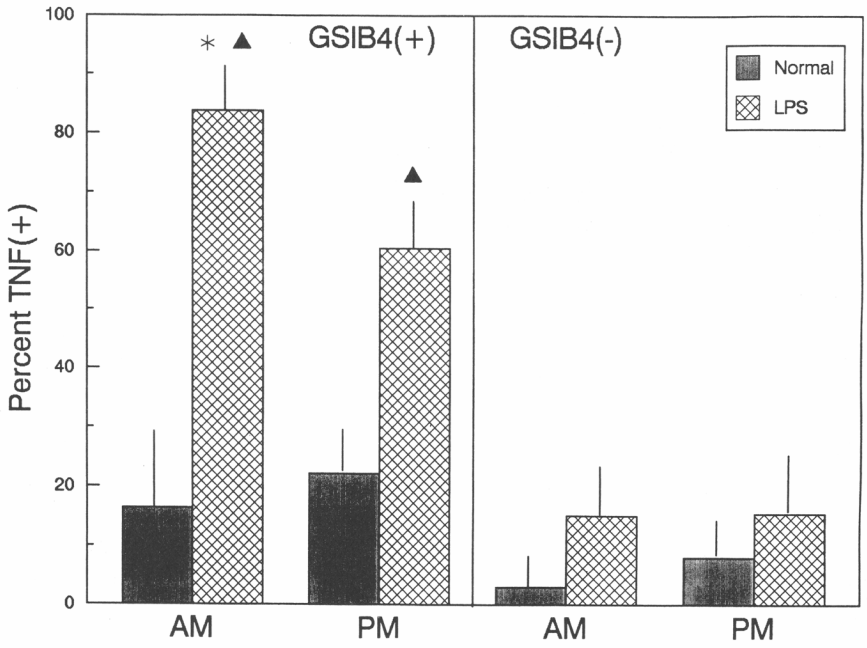

Figure 7. The expression of the $GSIB_4$-lectin-binding activity is differentially associated with alveolar and peritoneal macrophage TNF-α expression. Fresh explants containing AM and PM from unstimulated (normal) and LPS-stimulated animals were fractionated into $GSIB_4$(+) and (−) populations by cell column chromatography (see Materials and Methods). All cells were then washed and assayed for TNF-α expression by immunolocalization. The results are reported as the mean percentage positive ± SE. *Significantly different as compared with PM, $P < 0.001$. ▲, Significantly different as compared with $GSIB_4$(−) pair, $P < 0.001$.

Table II. TNF-α Secretion by $GSIB_4$ Positive and Negative Macrophages[a]

	TNF-α (pg/ml)	
	Alveolar macrophages	Peritoneal macrophages
$GSIB_4$ (+)		
Normal	181 ± 22	163 ± 37
LPS	1932 ± 49[b,c]	1590 ± 56[b]
$GSIB_4$ (−)		
Normal	90 ± 26	67 ± 18
LPS	174 ± 30	142 ± 27

[a] Alveolar or peritoneal macrophages from normal or LPS-treated rats were separated into $GSIB_4$ (+) or (−) fractions. Supernatants were collected and analyzed for TNF content. The results are reported in mean pg/ml ± SE.
[b] Significantly different as compared with $GSIB_4$ (−), $P < 0.001$.
[c] Significantly different as compared with peritoneal macrophages, $P < 0.001$.

no attempt was made to correlate marker expression and functional responses. Our studies, however, examined two macrophage activities and two anatomical compartments.

Thus, the data presented provide sufficient evidence to demonstrate that there is at least a concurrent relationship between the expressions of these two markers, both of which have functional value. It also shows that AM and PM differ in their magnitude of expression of these characteristics. Such results may be important for identifying activated macrophages capable of performing select functions. For example, $GSIB_4$ has the potential to be used as a marker for immunotherapy. By screening macrophages from patients with infectious or neoplastic processes for the expression of this marker, their activity level can be evaluated. Changes in this response as compared with normal healthy controls would provide information about the success of the treatment regimen and/or the status of the immune response at any given time. Although extensive work will be required to determine how these two events are related, the current results provide a basic understanding of the relationships between these markers and the macrophage.

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