ANTICANCER RESEARCH *24*: 2287-2294 (2004)

Involvement of Pattern Recognition Receptors in the Induction of Cytokines and Reactive Oxygen Intermediates Production by Human Monocytes/Macrophages Stimulated with Tumour Cells

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Abstract. *Background: Some ligands of pattern recognition receptors (PRR) are present on tumour cells. The role of PRR in signalling for cytokine and reactive oxygen intermediates (ROI) production by monocytes and monocyte-derived macrophages (MDM) stimulated with tumour cells was studied. Materials and Methods: Monocytes/MDM were pretreated with PRR ligands or anti-PRR monoclonal antibodies (mAbs) and stimulated with tumour cells. Cytokine secretion was measured by enzyme-linked immunoassay (ELISA) and ROI production by luminol-dependent chemiluminescence (CL). Results: The ligands of scavenger receptor A (SR-A): (fucoidan, polyguanylic acid (polyG) and modified low density lipoproteins (LDL)) and B (SR-B) (native and modified LDL, phosphatidylserine (PdS)) and of mannose receptor (MR) (mannan), induced tumour necrosis factor alpha (TNF) and ROI (except LDL) release by monocytes. Production of TNF and interleukin-10 (IL-10) by MDM was stimulated by SR-A ligands and mannan. Tumour cell-induced TNF and IL-10 production by monocytes, but not MDM, was diminished by fucoidan and polyG, while ROI release was reduced by MR and SR-A ligands. Supplementation of tumour cells with modified LDL and PdS enhanced their stimulatory capacity. TNF and ROI release by tumour cells-stimulated monocytes was inhibited by anti-CD36 and anti-MR (clone PAM-1) mAbs. Conclusion: SR and MR may be involved to different extents in the induction of cytokines and ROI production by monocytes, but not MDM, stimulated with tumour cells.*

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Key Words: Scavenger receptors, mannose receptors, monocytes, tumour cells, cytokines.

Macrophages possess the ability to distinguish transformed from normal cells. Hibbs (1) demonstrated that activated murine macrophages are able to recognize not only the cells with abnormal growth, but also to distinguish them from normal proliferating cells. C type lectins with galactoso/N-acetylgalactosamine (Gal/GalNAC) were indicated as being involved in this process (2, 3).

Human monocytes are also able to distinguish tumour from normal cells of the same histological origin and are cytotoxic ("spontaneous cytotoxicity") for the former. Tumour, but not normal, cells induce production of cytokines and reactive oxygen intermediates (ROI) by monocytes (4-6). Different membrane structures of tumour cells are implicated in the activation of human monocytes. These include: sialyl-LewisX–related carbohydrates (7), dGal-NAC (8) and phosphatidylserine (PdS) (9). The latter is also involved in the recognition of apoptotic cells by monocytes (10).

Among a large array of surface receptors expressed by monocytes/macrophages the pattern recognition receptors (PRR) like scavenger (SR), mannose (MR) or toll-like receptors (11) recognize the patterns of carbohydrates and lipoarabinomannan on the surface of microorganisms. In this way, in innate immunity the discrimination of self and non-self is achieved (12).

The scavenger receptors (SR) are multiligand receptors divided into several classes. Ligands of SR-A (both type I

and II) include modified low density lipoproteins (LDL), *i.e.* acetylated (acLDL) and oxidized (oxLDL), maleylated albumin, poly- and oligonucleotides (polyI, polyG), anionic polysaccharides (fucoidan, dextran sulphate) and lipopolysaccharide (LPS). SR-B type I and CD36 recognize native and modified LDL, maleylated albumin, PdS-containing vesicles and apoptotic cells (13, 14). Also mucins, the surface glycoproteins of tumour cells, interact with SR (15). The CD36 determinants are expressed mainly on monocyte/macrophages and foam cells, and are involved in the uptake and degradation of native and chemically-modified LDL, induction of proinflammatory cytokines and phagocytosis of apoptotic cells (13).

The MR belongs to a new family of receptors which bind mannose-, N-acetylglucosamine or fucose-terminated oligosaccharides, mediate the uptake of mucins (16) and are engaged in the recognition and internalization of apoptotic cells (17).

Since some ligands of PRR (PdS, mucins, carbohydrates) are present on tumour cells (7, 9, 18) we asked the question of whether these receptors are involved in monocyte-tumour cell interactions. Several ligands of PRR and monoclonal antibodies (mAb) against MR and CD36 were used for determination of the role of SR and MR in the induction of cytokine and ROI production by monocytes and monocyte-derived macrophages (MDM) stimulated with tumour cells.

Materials and Methods

Reagents. Fucoidan, mannan, LDL, polyG and PdS were purchased from Sigma (St. Louis, MO, USA) and used at the doses indicated in the Figures. MAbs against mannose receptor: MR (clone 19.2, IgG1) was obtained from BD Pharmingen (San Diego, CA, USA), MR 15-2-2 (IgG1) from Gaubius Laboratory (Leiden, The Netherlands) and PAM-1 (IgG1) was a kind gift from Dr. P. Allavena (Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy). Anti-CD36 (clone FA6-152) mAb was from Immunotech (Marseille, France) and anti-CD14 (clone MφP9) from Becton Dickinson.

LDL modification. Acetylated LDL (acLDL) was obtained by repeated additions of acetic anhydride to LDL using the protocol described by Basu *et al.* (19). Briefly, 16 mg of LDL in 1 ml of 0.15 M NaCl was added to 1 ml of solution of sodium acetate on an ice-water bath. Acetic anhydride was added in multiple small aliquots (2 μl) over a period of 1 h. The reaction solution was then dialysed for 24 h at 4˚C against buffer containing 0.15 M NaCl and 0.3 mM EDTA, pH 7.4. To prepare oxidized LDL (oxLDL), LDL (0.1 mg/ml) was incubated for 20 h at 37˚C with 5 μM $CuSO_4$ followed by addition of 1 mM EDTA and cooling (19).

Isolation of cell population. Human peripheral blood mononuclear cells (PBMC) were isolated from EDTA-blood of healthy donors by standard Ficoll/Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were separated from mononuclear cells by counter-flow centrifugal elutriation with a JE -5.0 elutriation system equipped with a 5 ml Sanderson separation chamber (Beckman, Palo Alto, CA, USA) as previously described (20). The cells were suspended in RPMI 1640 medium (Biochrom, Berlin, Germany) with gentamycin (25 μg/ml, Biochrom), glutamine (2 mM, Gibco, Paisley, UK) and 5% foetal calf serum (FCS, Biochrom), further referred to as complete medium. The purity of monocytes was in the range 90-96%, as judged by staining with anti-CD14 mAb (Becton Dickinson).

To obtain MDM, monocytes (10^6/ml) were plated in 6-well Ultra Low Attachment plates (Corning and Costar, Bodenheim, Germany) in 5 ml of complete medium per well. Every third day approximately 50% of the medium was removed and fresh medium was added. After 8 days of culture, cells were recovered by intensive pipetting, washed and suspended in the medium, as previously described (21).

Cell lines. HPC-4 (pancreatic adenocarcinoma) and DeTa (colorectal adenocarcinoma) were used as previously described (22). Cells were cultured by biweekly passages in RPMI 1640 with 5% FCS. Cell lines were regularly tested for *Mycoplasma* sp. contamination by Mycoplasma PCR ELISA test (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol.

Cell cultures. Monocytes or MDM (2 x 10^5/200 μl per well) were cultured in flat-bottom 96-well microtitre plates (Nunc, Roskilde, Denmark) in the presence of different doses of fucoidan, polyG and mannan (1-1000 μg/ml), PdS (0.1-10 μg/ml), native and modified LDL (0.1-100 μg/ml). After 18 h of incubation the supernatants were harvested and the level of cytokines was measured by ELISA. In another series of experiments, monocytes/MDM were preincubated with SR or MR ligands (30 min, 37˚C) or with mAbs at 1 μg/ml, final concentration, for 30 min at 4˚C, washed three times and then HPC-4 or DeTa tumour cells (at a ratio 1:0.3) were added and the cells were cultured for a further 18 h. In some experiments tumour cells were preincubated with PdS, native and modified LDL for 30 min at 37˚C, washed three times and used for monocyte stimulation.

Determination of cytokines. Concentrations of tumour necrosis factor alpha (TNF) and interleukin-10 (IL-10) in the culture supernatants were measured by commercial ELISA kits (Pharmingen, San Diego, CA, USA) according to the manufacturer's instruction. The detection level for TNF was 20 pg/ml and for IL-10 10 pg/ml.

Chemiluminescence. Monocytes (1 x 10^5) in 100 μl of culture medium with or without the ligands and 200 μl of 2 mM luminol (5 amino-2,3 dihydro-1,4 phtalazinedione, Sigma) in Krebs-Ringer buffer with Mg^{2+} and Ca^{2+} were added to the FluoroNunc 96-well plates (Nunc). The plates were placed in the measuring chamber of Victor2 (EG&G WALLAC, Turku, Finland) kept at 37˚C and the chemiluminescent (CL) response was continuously recorded. The results were expressed as integrals (cumulative counts, cc) of the response recorded during 300 min. In some experiments monocytes were preincubated with the ligands (30 min, 37˚C) or anti-receptor mAbs (1 μg/ml, 30 min, 4˚C) and, after washing, HPC-4 cancer cells (3 x 10^5/well) were added and CL measured.

Statistical analysis. Statistical analysis was performed by ANOVA. Differences were considered significant at p values < 0.05.

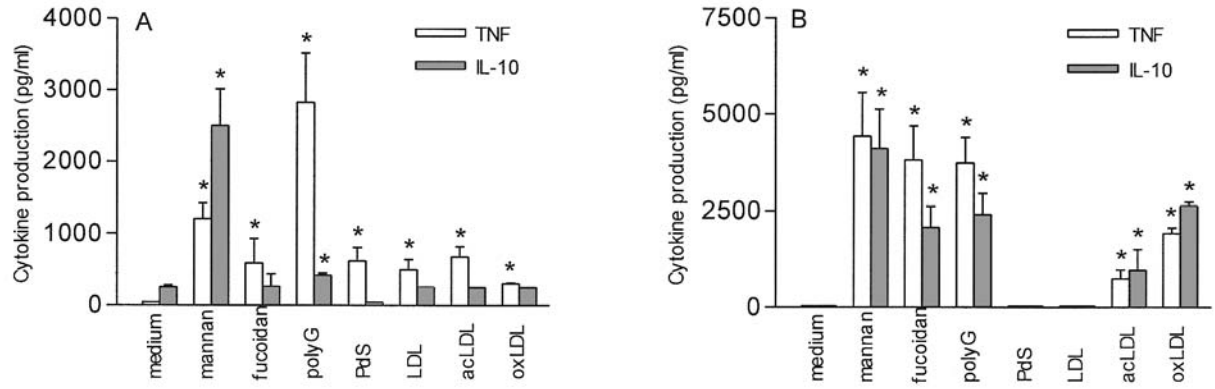

Figure 1. *Cytokine production by monocytes (A) and MDM (B) treated with different PRR ligands. The ligands were used at lowest active dose, i.e. mannan, fucoidan, polyG, native and modified LDL at 100 μg/ml, PdS at 1 μg/ml. Mean±SE of results obtained in 7 different experiments is shown. *statistically different ($p<0.05$) compared with untreated monocytes (medium).*

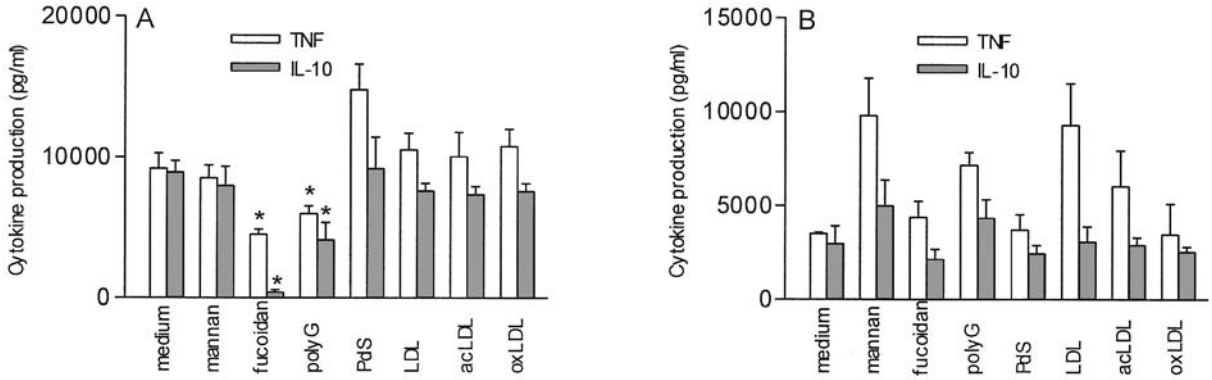

Figure 2. *Cytokine production by monocytes (A) and MDM (B) pretreated with PRR ligands in response to stimulation with tumour cells. Mean±SE of results obtained in 6 different experiments is shown. *statistically different ($p<0.05$) compared to the response of untreated monocytes/MDM co-cultured with tumour cells (medium).*

Results

Cytokine production by monocytes and MDM stimulated with different ligand of PRR. Monocytes were cultured for 18 h in the presence of fucoidan, polyG and modified LDL (SR-A ligands), PdS and LDL (SR-BI and CD36 ligand) and mannan (MR ligand). In the preliminary experiments different doses of ligands were tested and the lowest active doses (indicated in the figure legends) were employed in further experiments. Stimulation of monocytes with mannan and polyG led to a significant TNF secretion, while fucoidan, PdS, modified and native LDL induced little TNF release (Figure 1A). Among the ligands tested, only polyG slightly and mannan strongly induced IL-10 synthesis (Figure 1A). Mannan, fucoidan and polyG significantly stimulated TNF and IL-10 synthesis in MDM while modified LDL showed only a moderate stimulatory effect (Figure 1B). The level of cytokine production by MDM in response to these ligands was higher than by monocytes. PdS and LDL did not stimulate either TNF or IL-10. The above observations suggested that some polysaccharides (mannan, fucoidan), polynucleotides (polyG) and lipoproteins (LDL) but not phospholipids (PdS) may induce monocyte and MDM activation as measured by cytokine production. These results suggest that freshly isolated monocytes respond to MR, SR-A and SR-B ligands, while MDM to MR and SR-A ligands, and in both types of cells fucoidan and polyG more preferentially induce production of TNF than IL-10.

The effect of SR and MR ligands on cytokine production by monocytes and MDM stimulated with tumour cells. We have

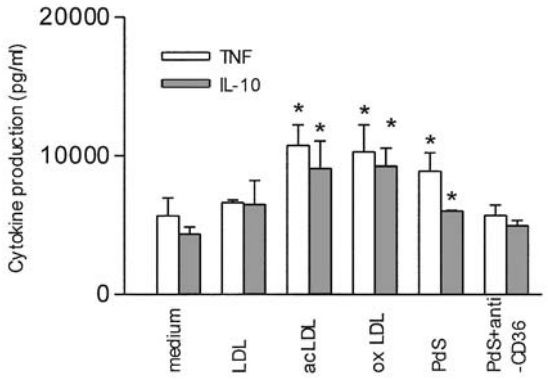

Figure 3. *Cytokine production by monocytes stimulated with tumour cells supplemented with native and modified LDL and PdS. Mean±SE of results obtained in 7 different experiments is shown. *statistically different ($p<0.05$) compared to the co-culture of monocytes with untreated tumour cells (medium).*

previously reported that several surface determinants and receptors participate in the interactions of monocytes with tumour cells, which results in the activation and production of cytokines, nitric oxide and ROI (5,6,20). In the present study, we asked whether SR and/or MR are involved in these interactions. To address this question, monocytes were pretreated with different SR or MR ligands and then stimulated with HPC-4 tumour cells for 18 h. Fucoidan and polyG efficiently inhibited TNF and IL-10 release by monocytes stimulated with tumour cells (Figure 2A). Both ligands suppressed more IL-10 than TNF (Figure 2A). Mannan did not inhibit tumour cell-induced cytokine synthesis. Unexpectedly, PdS, LDL, acLDL and oxLDL increased TNF and did not alter IL-10 production by monocytes stimulated with tumour cells (Figure 2A). The response of MDM to tumour cell stimulation was lower in comparison to monocytes. Of the ligands tested only fucoidan slightly diminished IL-10 synthesis by tumour cell-stimulated MDM (Figure 2B).

Cytokine production by monocytes stimulated with tumour cells supplemented with PdS, native and modified LDL. Previous experiments showed that PdS and modified LDL enhanced rather than blocked the response of monocytes to stimulation with tumour cells. To determine whether their enhanced expression on tumour cells might alter monocyte response, tumour cells were supplemented with exogenously supplied PdS, native and modified LDL. When the monocytes were stimulated with tumour cells labelled with PdS and modified, but not native, LDL an enhanced cytokine synthesis was observed in comparison to monocytes stimulated with untreated cells (Figure 3). Furthermore, the stimulatory capacity of PdS-labelled tumour cells was inhibited by anti-CD36 mAb, confirming the role of surface-bound PdS. This suggests that these ligands may be bound to tumour cells and enhance their ability to activate monocytes for cytokine production.

The effect of anti-SR and anti-MR mAbs. Pretreatment of monocytes with anti-CD36 mAb caused inhibition of TNF and no consistent inhibition of IL-10 production following stimulation with tumour cells (Figure 4A), pointing to the role of PdS and modified LDL in monocyte activation by tumour cells. To clarify the involvement of MR, three different anti-MR mAbs were used. Clone PAM-1, but not isotype-matched clones 19.2 and 15-2-2, inhibited TNF production by monocytes stimulated with tumour cells without apparent effect on IL-10 release (Figure 4A). Pretreatment of monocytes with both anti-CD36 and anti-PAM-1 mAbs did not increase the inhibition, suggesting a lack of synergistic effect. Neither anti-CD36 nor anti-MR mAbs affected tumour cell-induced cytokine production by MDM.

Induction of CL response of monocytes by PRR ligands and their role in ROI generation by monocytes stimulated with tumour cells. To determine the involvement of MR and SR in the induction of ROI production, their ligands were added to monocytes and CL was measured for a period of 300 min. Mannan, fucoidan, polyG, PdS and modified, but not native, LDL stimulated the CL response of monocytes (Figure 5A). The CL of monocytes stimulated with tumour cells was inhibited by mannan, fucoidan, polyG and by modified LDL. PdS and native LDL did not inhibit tumour cell-induced CL of monocytes (Figure 5B). Of the mAbs tested, anti-CD36 and anti-MR clone PAM-1, but neither 19.2 nor 15-2-2 clones, inhibited the tumour cell-induced CL of monocytes (Figure 5C). The data indicated the engagement of MR and SR in the tumour cell-induced CL response of monocytes, although the inhibitory effect of the ligands on ROI production was different from that on cytokine synthesis, *i.e.* fucoidan and polyG inhibited both responses while mannan and modified LDL affected CL only.

Discussion

Our previous data showed that contact with tumour, but not normal, cells activates monocytes and induces production of cytokines and ROI (5,6,20) and provided some evidence for the role of hyaluronan as the stimulatory molecule (22). However, tumour cells express or overexpress several other determinants that may be involved in signalling for monocyte activation. These may include: various carbohydrate structures, *e.g.* sialyl Lewis X-related (7), or mucins (16) and LDL, either synthesized by tumour cells or

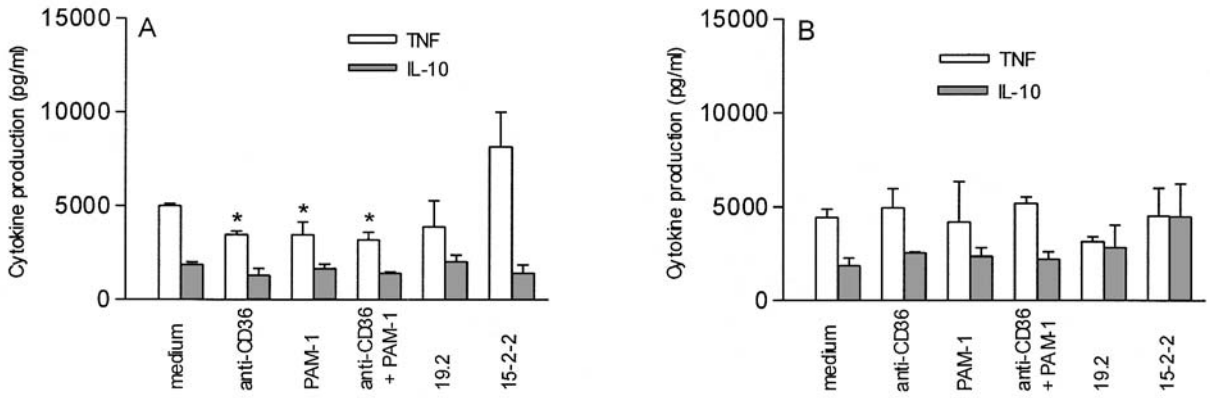

Figure 4. *The effect of anti-CD36 and anti-MR mAbs on cytokine production by monocytes (A) and MDM (B) following stimulation with tumour cells. Monocytes/MDM were pretreated with mAbs before addition of tumour cells. Monocytes/MDM pretreated with mAbs showed no cytokine release. Data (mean±SE) from 8 different experiments are presented. *statistically different ($p<0.05$) compared to monocytes/MDM not treated with mAb.*

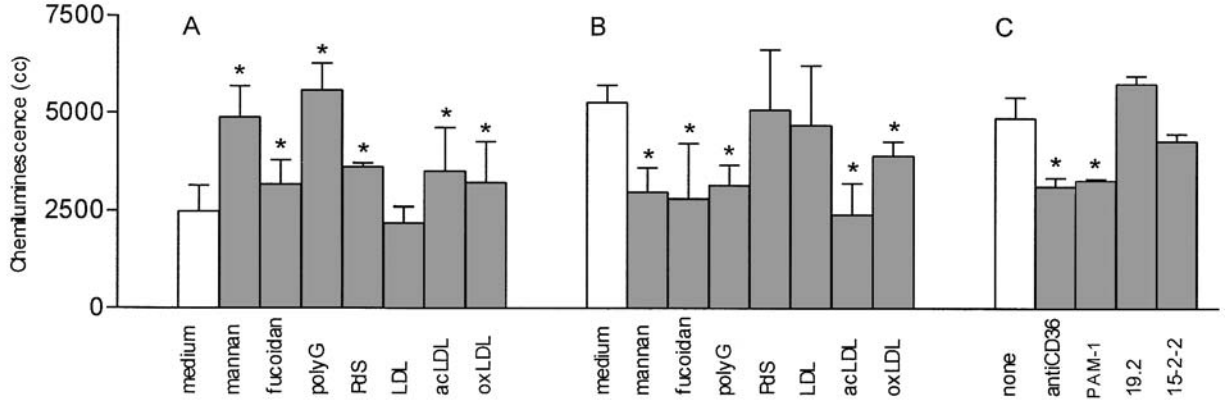

Figure 5. *CL of monocytes in response to PRR ligands (A) and of tumour cell-stimulated monocytes pretreated with PRR ligands (B) or anti-receptor mAbs (C). The results (mean±SE) of 8 different experiments are shown. *statistically different ($p<0.05$) compared to control (medium).*

acquired and bound to its receptor (23). Both monocytes and MDM produced TNF and IL-10 following stimulation with mannan, fucoidan and polyG (SR-A ligands). However, monocytes responded to native and modified LDL (SR-A and B), while MDM responded to modified LDL only (SR-A). The CL response of monocytes was induced by all ligands tested, except LDL, suggesting differences in the ability of SR ligands to evoke production of cytotoxic mediators. Production of ROI by MDM was not studied as they produce little ROI.

The other tumour cell surface structure which is recognized by monocytes/macrophages is PdS (9,24). PdS is present largely on the internal leaflet of plasma membranes of normal cells. Its exposure on the cell surface is connected with apoptosis, necrosis, cell activation or malignant transformation (25). There is also evidence that hypoxia and re-oxygenation may induce exposure of anionic phospholipids, most probably PdS, on the surface of tumour cells (25). Utsugi (9) reported that monocytes are able to distinguish tumourigenic cells expressing 3-7 times more PdS from non-tumourigenic cells with less PdS, suggesting its role in the recognition of tumour cells. However, in our hands soluble PdS was a poor inducer of TNF and did not stimulate IL-10 production, while tumour cells supplemented with PdS showed an enhanced ability to stimulate cytokine production by monocytes, indicating that PdS on the cell surface is probably recognized although presented in different molecular form. There is also evidence that crosslinking of CD36 evokes the CL response of monocytes, indicating that it acts as signalling molecule

(26). Our observations that PdS (CD36 ligand) induced the CL response of monocytes are in agreement with these findings. On the other hand, soluble PdS did not inhibit tumour cell-induced cytokine and ROI production by monocytes, though mAb against CD36 blocked these responses. These findings may suggest that surface-bound PdS on tumour cells is involved in their recognition by monocytes.

Fucoidan and polyG inhibited cytokine and ROI production by monocytes, implicating the role of SR-A in their interactions with tumour cells. However, these ligands did not affect the production of cytokines by MDM. This may be related to a higher expression of their receptors on MDM (27) resulting in inefficient blocking by the ligands. The other SR-A (*i.e.* modified LDL) and SR-B ligands (*i.e.* native LDL) and PdS did not interfere with tumour cell-induced cytokine production. On the contrary, an enhancing effect was seen suggesting that these ligands acted rather as agonists, hence their additive effect, than antagonists of SR.

Tumour cells express receptors for LDL which enable these cells to bind and internalize LDL (23). Along this line, we have pretreated tumour cells with native, modified LDL and PdS before addition to monocytes. The data show that tumour cells pre-exposed to PdS, oxLDL and acLDL, but not native LDL, show a substantially higher capacity to induce cytokine production by monocytes. Furthermore, anti-CD36 mAb inhibited the stimulatory effect of PdS-treated tumour cells, confirming the specificity of this effect. It appears to indicate that lipoproteins may not be sufficiently exposed on the surface of the tumour cells used in this study, however, when they are incorporated into the membrane or bound to the relevant receptor, may indeed be recognized by monocytes. This may be in keeping with their enhancing, rather than inhibitory, effect when used for monocyte pretreatment. Hence, this may implicate the role of modified (*e.g.* oxidized) LDL and, in consequence, confirms the role of SR-A in monocyte-tumour cell interactions, while the effect of PdS may suggests the role of CD36. Tumour cell-induced ROI production was also inhibited by mannan and modified, but not native, LDL. This apparent discrepancy with their effect on cytokine secretion may be due to a different turnover of the SR following binding the ligands as ROI production was studied over 300 min, while cytokine production after 18 h.

Although mannan did not inhibit tumour cell-induced TNF production by monocytes anti-MR mAb clone PAM-1, but not two other isotype-matched anti-MR mAbs (clones 19.2 and 15-2-2), efficiently blocked this and ROI production. It is of interest that PAM-1, but no other anti-MR (clone 19.2) mAb, inhibits IL-12 and enhances IL-10 production by LPS-stimulated monocyte-derived dendritic cells (28). This is compatible with our data on the inhibition of TNF production by tumour cell-stimulated monocytes, though we did not observe an enhancement of IL-10 production. However, in our hands no effect of any mAb on cytokine production by MDM was observed. This, and a lack of inhibitory effect of different PRR ligands on tumour cell-induced cytokine production by MDM, implies that neither SR nor MR are involved in tumour cell recognition by MDM.

In conclusion, we wish to suggest that several ligands of SR-A, SR-B and MR are able to stimulate cytokine and ROI release by monocytes and MDM and that different SR and MR are involved in the interactions of monocytes, but not MDM, with tumour cells resulting in cytokine and ROI production.

Acknowledgements

This study was supported by the State Committee for Scientific Research (grant 4 P05A 005 018), Poland.

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Received March 1, 2004
Accepted June 1, 2004