CD45-induced Tumor Necrosis Factor α Production in Monocytes Is Phosphatidylinositol 3-Kinase-dependent and Nuclear Factor-κB-independent*

(Received for publication, July 6, 1999)

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The pro-inflammatory cytokine tumor necrosis factor (TNF)α plays a pivotal role in the pathogenesis of rheumatoid arthritis. The mechanisms involved in regulating monocyte/macrophage TNFα production are not yet fully understood but are thought to involve both soluble factors and cell membrane contact with other cell types. Activation of certain cell surface receptors, namely CD45, CD44, and CD58, can induce the production of TNFα in monocytes. In this paper, we investigate further the signaling pathways utilized by cell surface receptors (specifically CD45) to induce monocyte TNFα and compare the common/unique pathways involved with that of lipopolysaccharide. The results indicate that monocyte TNFα production induced upon CD45 ligation or lipopolysaccharide stimulation is differentially modulated by phosphatidylinositol 3-kinase and nuclear factor-κB but similarly regulated by p38 mitogen-activated protein kinase. These results demonstrate that both common and unique signaling pathways are utilized by different stimuli for the induction of TNFα. These observations may have a major bearing on approaches to inhibiting TNFα production in disease where the cytokine has a pathogenic role.

Lipopolysaccharide (LPS)1 is one of the most potent activators of monocytes/macrophages, resulting in the triggering of a range of cellular responses and the secretion of pro- and anti-inflammatory cytokines, including TNFα, interleukin-1 (IL-1) and IL-6 (1–4). LPS, following interaction with serum proteins, e.g. LPS-binding protein and the cell surface receptor, CD14 (5), activates a number of signaling pathways. These include various tyrosine kinases (6, 7), protein kinase C (PKC) (8), the phosphatidylinositol 3-kinase (PI3K) pathway and that inhibitors of phosphatidylinositol 3-kinase (PI3K) (8, 9), p44/42 (extracellular signal-regulated kinase) (10), and p54 (stress-activated protein kinase/JNK) (11). Numerous studies have shown that direct contact between monocytes or monocytic cell lines and prestimulated T cells leads to production of cytokines including, IL-1β, TNFα, IL-12, and IL-10 (12–17). A variety of T cell-associated cell surface receptors/ligands including CD69, CD40L, CD11b, and CD2 are thought to be important in modulating this monocyte cytokine production (13–15). Furthermore, direct engagement of certain cell surface receptors, namely CD44, CD58, and CD45, on monocytes induce TNFα production (18, 19), suggesting that receptor engagement may be important in the regulation of cytokines. Potential ligands for CD45 (osteopontin) (20) and CD58 (CD2) (21) are expressed by activated T cells, whereas the ligand for CD45 still remains to be fully clarified, although the B cell adhesion molecule, CD22 (22), and the β-galactosidase-binding protein, galectin-1 (23), have been proposed to bind to specific isoforms of CD45.

CD45 is a membrane-anchored protein-tyrosine phosphatase found exclusively on all nucleated hemapoietic cells (24, 25). The role of CD45 in T cells has been the subject of much investigation and has been shown to play an important co-stimulatory role in intracellular signal transduction in T lymphocytes (26–31). While ligation of CD45 on monocytes has been shown to induce synthesis of cytokines, including TNFα, CD3, CD12, and macrophage-colony stimulating factor (M-CSF) (18, 19), the signaling mechanisms involved and the functional relevance of CD45 on monocyte/macrophages remain unclear.

We have investigated the signaling pathways utilized upon CD45 ligation on monocytes leading to TNFα production and compared this with the conventional stimulus, LPS. We demonstrate that CD45 ligation (but not LPS) activates the phosphatidylinositol 3-kinase (PI3K) pathway and that inhibitors of PI3K activation block CD45- but not LPS-induced TNFα synthesis. The differences in signaling also extended to nuclear factor-κB (NF-κB), which, unlike LPS, was not required by CD45-induced TNFα synthesis. In contrast, CD45, like LPS, activated p38 MAPK.

EXPERIMENTAL PROCEDURES
Reagents—LPS, wortmannin, and LY294002 were purchased from Sigma (Sigma, Poole, Dorset, United Kingdom (UK)). Rapamycin and SB203580 were purchased from Calbiochem-Novabiochem Ltd (Nottingham, UK). Human recombinant M-CSF was a generous gift from the Genetics Institute Inc. Phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P2 and phosphatidylserine (PtdSer) were purchased from Sigma, Poole, Dorset, UK). All reagents and medium used for monocyte culture were shown to contain <0.1 unit/ml endotoxin as measured using the Limulus amebocyte lysate assay (BioWhittaker).

Antibodies—Rabbit antisera to p38 MAPK was provided by Prof. J. Saklatvala (Kennedy Institute of Rheumatology, London, UK) (32) and the antibody to the p85α subunit of PI3K was kindly provided by Dr. D. Cantrell (ICRF, London, UK). The antibody to p70 86K was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phosphorylated protein kinase B (pPKB) and PKB were obtained from New England Biolabs (Hitchin, Herts, UK). Mouse IgG2a mAb HB196 (4B2 anti-CD45) and mouse isotype control IgG2a mAb OKT8 (anti-CD8) and OKT9 were obtained as hybridomas from ATCC, and antibodies were subsequently purified using a protein-G Sepharose column (Millipore,
Monocyte Purification—Peripheral human blood monocytes were isolated from single donor platelet-poor plasma purchased from the North London Blood Transfusion Service (Colindale, UK) as described previously (16). Briefly, mononuclear cells were isolated by Ficoll/ Hypaque separation (specific density 1.077 g/ml; Nycomed, A.S., Oslo, Norway), prior to cell separation in a Beckman J6e elutriator. Monocyte purity was assessed by flow cytometry using fluoro-chrome-conjugated anti-CD45 and anti-CD14 mAb (Becton Dickinson, Oxford, UK) and routinely consisted of >85% CD45- or CD14-expressing cells, respectively.

Culture—Monocytes were cultured in complete medium at 4 × 10^6 cells/ml in flat-bottomed 96-well culture plates (Nunc Life Technologies Ltd., Paisley, Scotland). At the start of the culture period, cells were either left unstimulated or were cultured with the following reagents as indicated in the text: 10 ng/ml LPS, 10 μg/ml immobilized anti-CD45 mAb or immobilized isotype-matched controls OX12 (IgG2a) and OKT8 (IgG2a). In some experiments monocytes were pre-treated for 15 min with wortmannin or LY294002, or for 1 h with SB203580 or rapamycin at the indicated concentrations prior to stimulation. After 18 h in culture at 37 °C with 5% CO₂, supernatants (200 μl/well, 3 wells/condition) were harvested and stored at −20 °C until used. All experiments were performed at least three times, and the figures represent representative examples of these experiments.

Adenoviral Infection—Cells were infected with an adenovirus encoding IκBα under the control of the cytomegalovirus promoter and a nuclear localization sequence (AdvIκBα) (36) or control adenovirus containing no insert (AdvΔ). Adenoviral infection of monocytes was performed as described previously (37). Briefly, freshly elutriated monocytes were cultured at 1 × 10^6/ml for 2–3 days with 100 ng/ml M-CSF. Following culture, M-CSF-treated monocytes were washed once with PBS to remove non-adherent cells and the remaining adherent monocytes were incubated with 10 μl of cell collagen solution (Sigma, UK) for 30–45 min to dissociate from the plastic. M-CSF-treated monocytes were resuspended to 2 × 10^6 cells/ml prior to stimulation with either 10 μg/ml immobilized anti-CD45 or 10 ng/ml LPS as indicated in the text for 18 h. Supernatants were harvested and assayed for TNFα production.

RESULTS

CD45 Induces Monocyte TNFα in Peripheral Blood Monocytes in a Concentration-dependent Manner—Fig. 1a illustrates TNFα synthesis following CD45 ligation on monocytes stimulated by immobilized anti-CD45 antibody in a concentration-dependent manner. There was also synergy between CD45 ligation and stimulation with LPS (10 ng/ml), as TNFα production was enhanced 4–6-fold (Fig. 1b) over that observed with LPS alone. In all experiments, immobilized isotype control antibodies did not induce TNFα production over that of cells alone.

Inhibition of PI3K Differentially Modulates Anti-CD45- and LPS-induced Monocyte TNFα Production—The signaling pathways involved in monocyte TNFα following CD45 ligation are unknown. In contrast, signaling pathways involved in LPS-induced TNFα production have received much attention. We have investigated the signaling pathways utilized upon CD45 ligation and compared these to LPS. Initial investigations focussed on PI3K, which is reported to be activated in monocytes upon LPS stimulation (38). Monocyte TNFα induced by anti-CD45 antibody (10 μg/ml) was inhibited in a dose-dependent manner by the PI3K inhibitor wortmannin (Fig. 2a) with an IC₅₀ of ~0.07 μM. In contrast wortmannin was found to synergize with LPS (10 ng/ml) to enhance TNFα production (Fig. 2b). To determine if the effects seen with wortmannin were due to inhibition of PI3K and not another signaling pathway, we studied the effects of another, structurally unrelated PI3K inhibitor, LY294002. LY294002, like wortmannin, was shown
to inhibit anti-CD45 antibody (10 \(\mu\)g/ml)-induced monocyte TNF\(\alpha\) production (IC\(_{50}\); 0.07 \(\mu\)M), while having little effect on LPS (10 ng/ml)-induced monocyte TNF\(\alpha\). (Fig. 2, c and d).

**CD45 Induces PI3K Activity in Peripheral Blood Monocytes**—
Due to the observed effects of the PI3K inhibitors, wortmannin and LY294002, we investigated PI3K activity. Engagement of CD45 on monocytes induced a transient increase in lipid kinase activity, maximal at 20 min and associated with immunoprecipitates of the anti-p85\(\alpha\) subunit of PI3K (Fig. 3). Treatment of these monocytes with wortmannin prior to stimulation with anti-CD45 antibody totally inhibited kinase activity (Fig. 3). In contrast, only a weak activation of PI3K was observed following LPS stimulation and none at all in control immunoprecipitates of isotype-matched monoclonal antibodies. Similar experiments with LY294002 were not possible, because unlike wortmannin, this compound does not covalently bind to the enzyme and thus is removed during the assay procedure.

**Ligation of CD45 Phosphorylates and Activates Downstream Effectors, PKB and p70 S6K**—Recent studies suggest that PI3K-mediated events are transduced via protein kinase B (PKB) (39). Ligation of CD45 resulted in phosphorylation of PKB with similar kinetics to that seen for activation of PI3K, and found to be maximal at 20 min. Fig. 4 illustrates PKB phosphorylation in monocytes following CD45 ligation, which was inhibited by pre-incubation with wortmannin or LY294002. In contrast, LPS induced only a weak phosphorylation of PKB, similar to that seen with the isotype control antibody. We next investigated the involvement of another known downstream effector of PI3K, p70 S6K (40). Ligation of CD45 on monocytes also resulted in activation of p70 S6K (Fig. 5), which was maximal at 30 min and was inhibited by pretreatment with rapamycin. Interestingly, however, the inclu-
sion of rapamycin did not inhibit anti-CD45-induced monocyte TNFα production (results not shown).

Anti-CD45-induced Monocyte TNFα Production Is NF-κB-independent—After demonstrating that TNFα production was differentially modulated by PI3K, we investigated the involvement of other factors known to regulate TNFα gene expression. We focused upon the transcription factor NF-κB, the activation of which has previously been shown to be important in TNFα production following LPS stimulation (37). Furthermore, it has recently been reported that NF-κB is activated by PI3K. Fig. 6a illustrates NF-κB binding activity following 30 min stimulation with LPS (0.1–10 ng/ml). Virtually maximal activation was observed with 1 ng/ml LPS, whereas in contrast anti-CD45 antibody (10 μg/ml) resulted in only a weak activation of NF-κB. It is unlikely that the difference in activation of NF-κB between LPS and CD45 ligation was simply due to a weaker stimulation provided by anti-CD45, because similar amounts of TNFα (750 pg/ml) were induced with anti-CD45 (10 μg/ml) and LPS (1 ng/ml) (Fig. 6b). These differences between LPS and CD45 ligation were further supported by the observation (Fig. 6c) that TNFα synthesis in LPS- but not anti-CD45-stimulated monocytes was inhibited by >80% when monocytes were infected with an adenoviral vector expressing the inhibitor of NF-κB (AdvIκBα).

Anti-CD45-induced TNFα Production Is Regulated by the
Signalling via CD45 in Monocytes

p38 MAPK Pathway—We have demonstrated that TNFα production in monocytes is differentially modulated by both PI3K and NF-κB. Numerous studies have demonstrated the importance of MAPKs, in particular the p38 MAPK, in LPS-induced TNFα production (9, 41). Therefore, we have investigated whether p38 MAPK is also involved in CD45-induced TNFα production, using an inhibitor of p38 MAPK, SB203580. SB203580 was found to inhibit both anti-CD45- and LPS-induced monocyte TNFα production, IC₅₀ values ~0.005 and 0.006 μM, respectively (Fig. 7, a and b). It has been previously demonstrated that LPS can activate p38 MAPK, with maximal stimulation seen at 10 min, followed by rapid loss of activation.² CD45 ligation also induced activation of p38 MAPK (maximal at 10 min) displaying similar kinetics to LPS (Fig. 8). Similarly, we have demonstrated that ligation of monocyte CD45 results in activation of p44/p42 MAPK with similar kinetics to LPS (results not shown).

DISCUSSION

In this paper we investigated the signaling pathway(s) involved in monocyte TNFα production following ligation of the cell surface receptor CD45 or LPS. Our results reveal the unexpected finding that CD45 ligation results in TNFα production that is dependent upon the activation of PI3 kinase but independent of the transcription factor NF-κB. In contrast, LPS-induced TNFα production was dependent upon NF-κB activation as previously reported (37) while PI3K-independent. These observations indicate that, while NF-κB has previously been shown to be important in TNFα production, it is not always necessary/required.

The importance of the cell surface receptor, CD45 in the activation of T and B cell antigen receptor-mediated signaling pathways and subsequent cellular responses has been well documented. Engagement of CD45 is known to regulate Src tyrosine kinases (p59fyn, p56fyn, and p70s6k) phosphorylation (42, 43), phospholipase Cγ1 regulation (44), inositol phosphate production (45), diacylglycerol production, PKC activation, and calcium mobilization (46). Ligation of CD45 has previously been shown to induce production of cytokines in monocytes (18, 19); however, the signaling pathways utilized upon CD45 ligation in monocytes have received little attention.

Ligation of monocyte CD45 results in activation of PI3K and the known downstream effectors PKB and p70 S6K. We have shown the anti-CD45-induced monocyte TNFα production is inhibited by the PI3K inhibitors, wortmannin and LY294002. However the inhibitor of p70 S6K activation, rapamycin, did not inhibit anti-CD45-induced TNFα production. These findings suggest that TNFα production is p70 S6K-independent and other, as yet unidentified, downstream components of PI3K pathway are involved.

In contrast, wortmannin but not LY294002 enhanced LPS-induced monocyte TNFα production, suggesting that the effects observed with wortmannin are not specific to PI3K activation. Wortmannin has other targets including PLA2 (47), and we have shown that the PLA2 inhibitor, AKTA, also enhances LPS-induced TNFα production in monocytes,² suggesting that the effect of wortmannin on LPS-induced TNFα production may be due to PLA2 inhibition. How PLA2 negatively regulates TNFα production is unclear, but this enzyme is required for synthesis of PGE₂, an inhibitor of TNFα production (48). Wortmannin is known to stimulate the stress-activated protein kinase pathway (49), and this may also have a positive effect on TNFα production. Furthermore, we observed only a weak increase in PI3K and p70 S6K activity following LPS stimulation, suggesting that neither of these pathways play a major role in LPS-mediated events in monocytes. These findings contradict those performed by Herrera et al. (38), in which LPS was demonstrated to induce PI3K activity in monocytes, using similar methods to those described here. The reason for these apparently contradictory findings remains unclear. These studies have focused upon class 1 PI3Ks, specifically those involving the p85α subunit and the involvement of other PI3K subclasses including those regulated by G-proteins and those which are wortmannin-insensitive have not been investigated.

p70 S6K and PKB are known downstream effectors of PI3K pathway—Activation of PI3K results in the phosphorylation of inositol phospholipids, leading to the activation of PKB and p70 S6K. We have shown that PKB and p70 S6K are involved in LPS-induced TNFα production, using an inhibitor of p70 S6K activation, rapamycin, which did not inhibit anti-CD45-induced TNFα production. These findings suggest that TNFα production is p70 S6K-independent and other, as yet unidentified, downstream components of PI3K pathway are involved.

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2 L. M. Williams and B. M. J. Foxwell, unpublished observation.

FIG. 7. SB203580 inhibits both anti-CD45- and LPS-induced monocyte TNFα production. 4 × 10⁶/ml monocytes were treated with SB203580 for 30 min prior to culture with immobilized anti-CD45 (10 μg/ml) (a) or LPS (10 ng/ml) (b) for 18 h. Supernatants were harvested and TNFα levels determined by ELISA. Results are expressed as the mean of triplicate cultures ± S.D. This figure is representative of five experiments performed using different donors. In all experiments immobilized relevant isotype control (10 μg/ml) did not induce TNFα production (results not shown).

FIG. 8. CD45 ligation and LPS stimulation of monocytes activates p38 MAPK. 5 × 10⁶ monocytes were cultured with LPS (10 ng/ml), immobilized anti-CD45 (10 μg/ml), or isotype control (IC) antibodies (10 μg/ml) for given times. Postnuclear lysates were incubated with a suspension of protein G and anti-p38, p38 MAPK activity was assessed via [γ-32P]ATP incorporation into ATF-2. Phosphorylated products were visualized by autoradiography using Hyperfilm.
duced TNFα production in monocytes is p70 S6K-independent. Furthermore, while ligation of CD45 induces phosphorylation of PKB, the involvement of PKB in monocyte TNFα production at this stage cannot be verified due to the lack of specific PKB inhibitors. These findings indicate that there must be a bifurcation of the signaling pathways downstream of PI3K that regulate TNFα production. Several signaling molecules have been shown to directly and/or indirectly regulate PI3K, leading to the activation of transcription factors, e.g., atypical PKCζ and PKCα (52). Unfortunately, inhibitors of PKC were found to be toxic to monocytes and as such the involvement of PKC in anti-CD45-induced TNFα production has not been assessed. Other potential downstream effectors include Rac, Rab5 (53, 59), Bruton's tyrosine kinase (55, 56), and JNK/stress-activated protein kinase (57, 58). The involvement of these molecules in PI3K-dependent TNFα production still remains to be determined.

Several studies have suggested that LPS-induced TNFα production in monocytes/macrophages is NF-κB-dependent. Protease inhibitors, gliotoxin, and free radical scavengers have all been used to block NF-κB activity; however, the lack of specificity of these reagents remains a constant problem. More recently, the over expression of IkBα following adenoviral infection (AdvIkBα) has been demonstrated to inhibit LPS-induced TNFα production in monocytes (37). Curiously, we demonstrated that ligation of CD45 induced IkBα degradation (results not shown) but only a weak NF-κB binding activity; the reasons for this remain unclear, although it suggests further complexity of the NF-κB system. Overexpression of AdvIkBα did not inhibit anti-CD45-induced TNFα production. These findings indicate that other, as yet unidentified, transcription factors are involved in anti-CD45-induced TNFα production.

In T cells, induction of TNFα gene expression is regulated by the nuclear factor of activated T cells (NFAT), not NF-κB (59, 60, 61). NFAT binds to the κ3 element of the TNFα gene (located −97 and −88 nucleotides relative to the TNFα start site), in association with ATF-2 and c-Jun proteins, which bind to the cyclic AMP response element site (62). NFAT DNA binding activity in activated T cells is prevented by the immunosuppressive drugs cyclosporin A (CsA), and FK506 (63, 64, 61). CsA and FK506 form complexes with their intracellular binding activity in activated T cells is prevented by the immunosuppressive drugs cyclosporin A (CsA), and FK506 (63, 64, 61). CsA and FK506 form complexes with their intracellular receptors (immunophilins), and inhibit the activity of calcineurin (protein phosphatase 2B), a ubiquitous calcium- and calmodulin-dependent phosphatase (reviewed in Ref. 65). Induction of TNFα mRNA gene transcription in T cells can be blocked by CsA and FK506 (62), and expression of calcineurin is sufficient to activate a reporter gene whose transcription is driven by the TNFα promoter (60). The involvement of NFAT in monocyte TNFα production remains to be confirmed. However, CsA and FK506 failed to inhibit anti-CD45-induced TNFα production in monocytes (results not shown), but this does not discount the involvement of CsA-insensitive NFAT in the regulation of monocyte TNFα production. These findings suggest that NFAT, like NF-κB, is not required for anti-CD45-induced TNFα production.

LPS has previously been shown to activate the three major mammalian MAPK pathways, p42/44 (extracellular signal-regulated kinases 1/2), p38, and p54 MAPK (stress-activated protein kinase), in monocyte/macrophages (9, 10, 11). However, the relationship between the activation of these signaling molecules cytokine expression remains to be clarified. p38 MAPK is the only kinase that has been shown to play a pivotal role in the production of TNFα (66). Previous studies have suggested that the post-transcriptional regulation of TNFα is mediated through adenosine-uridine (AU)-rich elements present within the 3′-untranslated region of the TNFα mRNA (67). Deletion of this region leads to the constitutive synthesis of TNFα in cells lines (68) and transgenic animals (69). TNFα reporter gene constructs that do not contain the 3′-AU-rich element regions lose their sensitivity to inhibition by the p38 inhibitor, SB203580, and it has been suggested that the p38 MAPK cascade is mediating the release of translational repression of TNFα (66). The pyridinyl imidazole compound, SB203580, has been used to determine the involvement of p38 MAPK in the regulation of numerous pro-inflammatory cytokines including IL-1, IL-6, and TNFα (9). Recently, SB203580 has been shown to inhibit TNFα protein and mRNA induced by LPS, suggesting that TNFα is being inhibited at the pre-translational level (70, 71). We have demonstrated that monocyte TNFα production is regulated by distinct transcriptional mechanisms. Furthermore, we have demonstrated that both LPS- and anti-CD45-induced TNFα production is regulated by p38 MAPK suggesting that both stimuli utilize similar translational mechanisms to regulate TNFα production. We observed that ligation of CD45 resulted in activation of the MAPks p38 and p42/p44 (results not shown) with similar kinetics to that observed with LPS. Furthermore, inhibitors of p38 MAPK (SB203580) and p42/44 MAPK (PD98059) (results not shown) were shown to block both anti-CD45 and LPS-induced TNFα production. At higher concentrations SB203580 is known to inhibit the activity of JNK2 and JNK3 (72); however, the IC50 values observed for SB203580 inhibition of anti-CD45- and LPS-induced monocyte TNFα synthesis are consistent with its effects on p38 MAPK and not JNK, although the nonspecific actions of this drug cannot be disregarded. These findings indicate that TNFα production is regulated by distinct transcriptional signaling mechanisms, while the translational mechanisms appear to be identical.

In summary, this study demonstrates that TNFα production in monocytes is regulated by multiple signaling pathways. The initiating signals for TNFα production in inflammatory disorders such as rheumatoid arthritis are unknown. However, these findings suggest that engagement of specific cell surface receptors may be important in regulating TNFα production via distinct signaling pathways and investigation of these mechanisms in both physiological and pathological systems is currently being investigated.

Acknowledgment—We thank Dr. C. Ciesielski for assistance with adenosine infection of monocytes.

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