Experimental studies on signal transduction pathways in rheumatoid arthritis

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Monocytes and monocyte-derived macrophages differ in regulation of signal transduction pathways

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In preparation
ABSTRACT

Background. In rheumatoid arthritis (RA) macrophages play a key role in the inflammatory process. It has been reported that inhibitors of signal transduction pathways interfere with cell differentiation. As p38 MAPK (mitogen-activated protein kinase) inhibitors are now in phase II clinical trials in RA, it is relevant to know whether these inhibitors differ with respect to their effects on monocytes and macrophages, and whether they influence the differentiation process.

Objective. Aim of the study was to investigate whether p38 MAPK inhibition has a different effect on mediators produced by LPS-stimulated monocytes or monocyte-derived macrophages (MDM). In addition we investigated whether p38 MAPK inhibition (by RWJ 67657) influences the process of differentiation of monocytes into macrophages.

Methods. Monocytes and MDM were stimulated with 50 ng/ml LPS with or without pretreatment of 1 µM RWJ 67657. IL-1β, TNF-α, IL-8 and MMP-9 mRNA expression and protein production were measured with real-time RTPCR and ELISA. Furthermore, monocytes were left to differentiate in the presence of 0, 0.1 or 1 µM RWJ 67657. Cells were stimulated with LPS at day 1, 2, 3, 4, and 5, and cytokine and MMP-9 mRNA and protein levels were measured. Lysates of stimulated and unstimulated monocytes and MDM were also tested in a kinase array to identify which kinases were activated.

Results. Monocytes produced more cytokines than MDM, but less MMP-9. Inhibition with the p38 MAPK inhibitor RWJ 67657 was more effective in monocytes than in MDM. Differentiation of monocytes into macrophages in the presence of the p38 MAPK inhibitor significantly reduced TNF-α, IL-8 and MMP-9 protein production by macrophages.

Conclusions. It was demonstrated that monocytes respond better to p38 MAPK inhibition than monocyte-derived macrophages, and that differentiation is influenced by p38 MAPK inhibition. With kinome profiling we found that several kinases involved in the p38 MAPK pathway are activated both in stimulated monocytes and MDM. Differences in kinase activity were found between monocytes and MDM, that need further investigation.
INTRODUCTION
Macrophages are known to play an important role in inflammatory diseases such as rheumatoid arthritis. Rheumatoid synovium is intensively infiltrated by macrophages and their numbers correlate well with articular destruction and clinical scores. Macrophages perform many functions including phagocytosis, killing of pathogens, and the release of cytokines, proteinases and other inflammatory mediators. In response to inflammatory conditions, monocytes migrate from the bone marrow into the vasculature, and subsequently into extravascular tissues. Transmigration of monocytes is accompanied by changes in morphological, biochemical, and functional characteristics. Circulating monocytes possess a markedly different functional phenotype as compared to tissue macrophages. It has been demonstrated that the route of monocyte differentiation determines their cytokine production and phenotype expression. Two cytokines that have been indentified as promoters of monocyte differentiation are macrophage-colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF). In vitro maturation of monocytes into macrophages by M-CSF with only 1% FCS has been shown to yield a maximum of macrophages without inducing proliferation or activation. CSFs have been found to be present at elevated levels in the synovial fluid of RA patients. Also in collagen-induced arthritis models in mice, M-CSF has been shown to exacerbate the arthritis. Because of the crucial role synovial macrophages have in the inflammatory process in RA, they are target of therapy themselves, for instance by clodrate-containing liposomes and recently by anti-FcγRI (CD64) antibody to which the plant toxin ricin A (RTA) was chemically linked.

In vitro stimulation of monocytes and macrophages by a variety of agents causes activation of signal transduction pathways and in particular activation of mitogen-activated protein kinases (MAPK). Cytokine production by monocytes and MDM has been reported to be significantly reduced by treatment with specific p38 MAPK inhibitors. Stimulation with lipopolysaccharide (LPS) is not only regulated by CD14 but also involves Toll-like receptors, leading to activation of stress-induced signal transduction pathways. Differentiation of macrophages with serum is reported to be under influence of the p38 MAPK pathway. In monocytic cell lines differentiation was shown to be potentiated by p38 MAPK inhibitors, with concomitant up-regulation of the c-jun N-terminal kinase (JNK) pathway. Between 10-15% of the proteins encoded by the human genome are involved in intracellular signal transduction. Nearly a third of all the different types of proteins inside the cells are subject to phosphorylation, usually at multiple sites. Recently progress has been made with the preparation of arrays exhibiting specific consensus sequences for protein kinases. This kind of array would allow faster and more extensive analysis of activity of various intracellular signaling pathways. Usefullnes of peptide arrays was recently tested by Diks et al., who demonstrated that with this array the enzymatic activities of a large group of kinases could be determined in LPS-stimulated PBMC.

In this study we investigated whether p38 MAPK inhibition had a different effect on mediators produced by LPS-stimulated monocytes or monocyte-derived macrophages. Furthermore we investigated whether a p38 MAPK inhibitor (RWJ 67657) did...
influence M-CSF-induced differentiation of monocytes into macrophages. Finally we tested stimulated and unstimulated monocytes and MDM in the kinase array.

MATERIAL AND METHODS

Reagents
The p38 MAPK specific inhibitor RWJ 67657 was provided by Johnson and Johnson (R.W. Johnson Pharmaceutical Research Institute, Raritan, New Jersey, USA). Recombinant human macrophage colony stimulating factor (M-CSF) and ELISA antibodies were from R&D Systems (Minneapolis, Minnesota, USA). Foetal calf serum (FCS) and RPMI 1640 culture medium were obtained from Biowhittaker (Verviers, Belgium). All reagents for RNA isolation and reverse transcriptase reaction were purchased from Invitrogen, Life Technologies (Gaithersburg, Maryland, USA). Reagents for real-time RT-PCR were obtained from Applied Biosystems (Foster City, California, USA).

Macrophage culture
Blood was obtained from apparently healthy laboratory workers. Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep density gradient centrifugation from citrated blood. Cells were suspended in RPMI with gentamycin at 2.10⁶ cells/ml and seeded in 3 ml in 6-well plates (Corning, Schiphol, The Netherlands) or 0.5 ml in 24-well plates and cultured at 37°C in a 5% CO₂ atmosphere. The cells that adhered after two hours were used as monocytes or were allowed to differentiate into monocyte-derived macrophages (MDM) for five days in RPMI containing gentamycin, 50 ng/ml M-CSF + 1% FCS, while medium was refreshed at day two.

Experimental set-up
To investigate whether unstimulated or LPS stimulated monocytes and MDM responded differently to treatment with p38 MAPK inhibition, cells from four donors before and after differentiation were stimulated with 50 ng/ml LPS, with or without one hour pre-treatment with 1 µM RWJ 67657 during four hours for mRNA expression measurement or 24 hours for protein determination. After four hours stimulation cells were lysed in TRIzol reagent according to the manufacturers instructions (Life Technologies) and stored at -80°C until RNA isolation. For protein determination supernatants of treated cells were harvested and stored at -20°C until measurement.

The effect of p38 MAPK inhibition on cell differentiation was investigated by incubating cells from four donors with 0, 0.1 or 1.0 µM RWJ 67657 during the entire differentiation period. At day 1, 2, 3, 4 and 5 cells were stimulated with 50 ng/ml LPS for four or 24 hours as described above. Before LPS stimulation RWJ 67657 was depleted from the medium by washing for 30 minutes, which experimentally has been shown to be sufficient (data not shown).

RNA isolation and real-time RT-PCR
RNA isolation was performed using TRIzol reagent according to the manufacturers instructions and mRNA expression of IL-1β, TNF-α, IL-8, MMP-9, and glyceralde-
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hyde-3-phosphate dehydrogenase (GAPDH), 1µl of cDNA in triplicate was used for amplification by the real-time quantitative PCR system (ABI Prism 7900HT Sequence Detection System, Applied Biosystems) with specific Taqman primers/probes as described earlier. In our experiments GAPDH and other genes of interest were always determined in the same RT-PCR run. Amplification was performed using standard conditions: denaturation at 95°C for 15 seconds, 40 cycles of amplification with annealing at 60°C for 1 minute, and extension at 50°C for 2 minutes. According to the comparative Ct (threshold cycle value) method described in the ABI manual, the resulting mRNA amount of the gene of interest was normalized to the housekeeping gene GAPDH, yielding the \( \Delta \)Ct value. The \( \Delta \)Ct value of unstimulated cells was subtracted from the average \( \Delta \)Ct value of each sample, yielding the \( \Delta \Delta \)Ct. The amount of target, normalized to an endogenous reference (GAPDH) and relative to the control sample, is given by: \( 2^{-\Delta \Delta \text{Ct}} \).

ELISA based determination of IL-1β, TNF-α, IL-8, and MMP-9 in cell culture supernatants

Cytokine (TNF-α, IL-1β, and IL-8), and MMP-9 levels were measured in cell supernatants by ELISA, using matched antibody pairs for ELISA and recombinant proteins as standards from R&D Systems. Detection limits for all cytokine ELISAs was 50 pg/ml and for MMP-9 ELISA 100 pg/ml.

Kinome profiling with kinase array

To compare the activity of kinases between monocytes and MDM, kinase arrays were performed as described earlier.

First it was investigated by western blotting whether monocytes were already activated by the isolation procedure and the adherence. Previously it was shown that unstimulated MDM had low phospho-p38 MAPK activity. PBMCs were isolated as described above and were left to adhere in 6-well plates at a concentration of 2.10^6 cells/ml (3 ml/well) in RPMI + 1% FCS. After 1, 2, 4, and 6 hours cells were either activated with 50 ng/ml LPS for 30 minutes and then lysed in 300 µl lysis buffer or were lysed unstimulated. Lysis buffer contained 2% SDS, 10% glycerol, 50 mM dithiothreitol, 62.5 mM Tris-HCl (pH=6.8) and 0.01% brom-phenol blue. Cells were scraped off the wells and the lysates were subsequently sonicated for 5-10 seconds and boiled for 5 minutes. After centrifugation the samples were loaded onto a 10% SDS-PAGE gel and resolved by running at 200 V and 15 Watt constant. Semidry blotting was performed onto nitrocellulose membrane and immunodetection with anti-phospho-p38 MAPK was performed, followed by incubation with peroxidase-anti-rabbit IgG. Enhanced chemi-luminescence (ECL) detection was performed according to the manufacturers guidelines (Lumi-Lightplus, Roche Diagnostics, Mannheim, Germany). Blots were exposed to Hyperfilm™ (Amersham Biosciences, Roosendaal, The Netherlands) and developed. Subsequently, blots were stripped with Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) and immunodetection with anti-p38 MAPK was performed.
Chapter 5

Peptide array analysis
For kinase array 10^7 PBMC/well were plated in 4 ml RPMI + 1% FCS in 6-well plates, and were left to adhere or were differentiated to MDM with M-CSF for five days as described above. The following experiment was performed on both monocytes (after two hours of adherence) and MDM: one well was left unstimulated, one well was stimulated with 50 ng/ml LPS for 30 minutes. Stimulations were finalized by washing with ice-cold phosphate buffered saline. Next, cells were lysed in ice-cold kinase lysis buffer (100 µl/well): 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 mM MgCl_2, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM PMSF. The cell lysates were centrifuged at 10000 rpm for ten minutes. Peptide array incubation mix was produced by adding 10 µl of filter-cleared activation mix (50% glycerol, 50 µM [γ-33P]ATP, 0.05% v/v Brij-35, 0.25 mg/ml bovine serum albumin, [γ-33P]ATP (1000 kBq) to 50 µl of cell lysate. The lysate + peptide array mix was added onto the chips which consists of kinase substrate peptides on a glass slide (Pepscan®kinase, Pepscan Systems, Lelystad, The Netherlands) and the chips were kept at 37°C in a humidified stove for 75 minutes. The chips contain 1176 kinase substrate peptides in duplicate, and the consensus substrate peptides are approximately 9 peptides long. Subsequently the chips were washed twice with phosphate-buffered saline + 0.1% Triton, twice in 2 M NaCl, twice in demineralized H_2O and then air dried. The chips were then exposed to a phosphor-imager plate for 72 hours, and the density of the spots was measured and analyzed with array software (ScanAlyze, Stanford University, Stanford, California, USA).

STATISTICS
Paired T-tests were performed using GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, CA, USA).

RESULTS
Effects of p38 MAPK inhibition on mediator production by monocytes and monocyte-derived macrophages
In table 1 the protein production (IL-1β, TNF-α, IL-8 and MMP-9) by monocytes and MDM before and after LPS stimulation and 1 µM RWJ 67657 pre-treatment is listed. The table shows that unstimulated monocytes did not produce TNF-α and IL-1β, and low IL-8 levels, whereas high levels were produced after LPS stimulation. Unstimulated MDM did not produce TNF-α and IL-1β, and low IL-8, but in contrast to unstimulated monocytes, produced unstimulated MDM high levels of MMP-9. In all situations there was inhibition due to pretreatment with 1 µM RWJ 67657, but inhibition appeared to be more effective in monocytes than in MDM. Except for unstimulated monocytes MMP-9 production was less effectively inhibited compared to cytokines. mRNA expression was measured in stimulated monocytes and MDM, and mRNA expression in unstimulated cells was used as control (fold induction =1, table 2).
Differences between monocyte and MDM signal transduction

In contrast to protein expression IL-1β mRNA was highly expressed in stimulated MDM. All cytokine genes were expressed at a higher level in MDM than in monocytes. MMP-9 mRNA was constitutively expressed in both monocytes and MDM and expression was not induced by stimulation (fold induction < 2). Again p38 MAPK inhibition was effective for cytokine mRNA expression, but not for MMP-9 expression.

| Table 1. Protein production by monocytes and MDM with and without pre-treatment of 1 µM RWJ 67657. Cells were unstimulated or stimulated for 24 hours with 50 ng/ml LPS. Protein concentration of cytokines and MMP-9 was measured in supernatants by ELISA. BD = below detection, inh = % inhibition. |
|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
|                                   | Monocytes, unstimulated            | MDM, unstimulated                | Monocytes, stimulated            | MDM, stimulated                  |
|                                   | RWJ Mean (SEM) Inh                 | RWJ Mean (SEM) Inh               | RWJ Mean (SEM) Inh               | RWJ Mean (SEM) Inh               |
|                                   |                                   |                                   |                                   |                                   |
|                                   | - BD                               | BD                                | 2392(421)                        | BD                                |
|                                   | + BD                               | BD                                | 331 (63)                         | 86% BD                           |
|                                   | - BD                               | BD                                | 2021(374)                        | 1075(493)                        |
|                                   | + BD                               | BD                                | 138 (39)                         | 93% 587(305)                     | 45% |
|                                   | - 1.7(0.6)                         | 0.3(0.1)                         | 116 (69)                         | 11.7 (1.7)                       |
|                                   | + 0.03(0.01)                       | 98%                              | 0.08(0.02)                       | 74% 51.2(23.7)                   | 56% 6.9(1.3)                     | 41% |
|                                   | - 6.2(2.1)                         | 79.8(19.2)                       | 28.6(10.1)                       | 97.0(13.5)                       |
|                                   | + 1.1(0.5)                         | 82%                              | 52.3(13.0)                       | 34% 22.7(9.1)                    | 21% 61.4(11.0)                   | 37% |

| Table 2. mRNA expression (fold induction) in stimulated monocytes and MDM with and without pre-treatment of 1 µM RWJ 67657. Cells were stimulated with 50 ng/ml LPS for 4 hours and mRNA expression of cytokines and MMP-9 was determined by real-time RT-PCR. |
|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
|                                   | Monocytes                         | MDM                              |
|                                   | RWJ Mean (SEM) Inhibition         | RWJ Mean (SEM) Inhibition         |
|                                   |                                   |                                   |                                   |                                   |
| IL-1β                             |                                   |                                   |                                   |                                   |
| -                                  | 341.6(140.9)                      | 3434.0(1423.2)                    |                                   |
| +                                  | 84.9(42.9)                        | 1093.5(549.4)                     | 68%                              |
| TNF-α                             |                                   |                                   |                                   |
| -                                  | 175.7(28.0)                       | 589.7(158.6)                      |                                   |
| +                                  | 33.5(16.0)                        | 125.2(33.9)                      | 79%                              |
| IL-8                              |                                   |                                   |                                   |
| -                                  | 67.8(30.2)                        | 324.2(118.9)                      |                                   |
| +                                  | 15.3(10.0)                        | 207.9(89.6)                      | 36%                              |
| MMP9                              |                                   |                                   |                                   |
| -                                  | 1.10(0.33)                        | 1.50(0.12)                      |                                   |
| +                                  | 0.65(0.25)                        | 1.33(0.43)                      | 11%                              |
Figure 1. Protein production by monocytes and MDM during differentiation in the presence of 0, 0.1, and 1.0 μM RWJ 67657. Cells were unstimulated or stimulated for 24 hours with 50 ng/ml LPS. Protein concentration of cytokines and MMP-9 was measured in supernatants by ELISA (* p<0.05, ** p<0.01, *** p<0.001, paired T-test, tested against MDM, untreated with RWJ 67657).

Effects of p38 MAPK inhibition on differentiation
As p38 MAPK inhibition might not only affect protein production and mRNA expression in cells but also might have an effect on the differentiation process, we left monocytes to differentiate in the presence of 0, 0.1 and 1.0 μM RWJ 67657. In figure 1 the protein production of cytokines and MMP-9 is shown in unstimulated and stimulated cells during the differentiation process. Figure 1 shows clearly that immediately after differentiation has started IL-1β production was decreased below detection limit of the assay, while TNF-α production was decreased by more than 70%. For all cytokines the presence of RWJ 67657 reduced the production during
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MMP-9 production was increased during differentiation, and the presence of RWJ 67657 led to reduced protein production. Measurement of mRNA expression (figure 2) confirmed the protein data concerning reduced expression in cells differentiated in the presence of the p38 MAPK inhibitor.

Figure 2. mRNA expression of TNF-α, IL-1β, IL-8, and MMP-9 in stimulated monocytes and MDM during differentiation in the presence of 0 and 1 µM RWJ 67657.

Kinome profiling of monocytes and MDM

To investigate the activation state of isolated and adhered monocytes, cell lysates were prepared from monocytes after isolation and different adherence time points. In figure 3 the results are shown of western blots with antibodies to p38 MAPK and phosphorylated p38 MAPK. After one hour of adherence the cells were still activated and showed phospho-p38 MAPK reactivity, but after two hours of adherence the activity was decreased. Cells were also stimulated with 50 ng/ml LPS for 30 minutes as control, which gave strong phospho-p38 MAPK bands. Next cell lysates were prepared of monocytes after two hours of adherence and of MDM, unstimulated and stimulated for 30 minutes. Kinase arrays were performed with the lysates, and radioactivity bound to the chips was detected with phosphorimager. With the ScanAlyze program the spots were quantified, and the correlations between the duplicates after correction for background were calculated. Correlations between the duplicates were well above 0.70, except for the kinase chip of the unstimulated monocytes, of which only one of the duplicates could be used.
Figure 3. Western blot of monocytes after different timepoints of adherence. Activation status of cells was measured with phospho-p38 MAPK, p38 MAPK was used as control.

In Table 3 results are shown of kinases involving in the p38 MAPK pathway. The results under control and LPS represent the radioactivity incorporated in the substrates (in Phosphorimager/Aida quantification units) and are the average of 2 measurements, except for the unstimulated monocytes results. The ratio of LPS stimulated samples and controls is given in the next column. From the results it appears that all substrates involved in the p38 MAPK pathway are phosphorylated after stimulation with LPS, although differences in intensity are seen between monocytes and MDM.

Table 3. Monocyte and MDM kinase activity. Depicted are kinases and substrate involved in the p38 MAPK pathway. Results represent the radioactivity incorporated in the substrates (in phosphorimager/Aida quantification units). (MK2-MAPKAPK2, cPLA2-cytosolic phospholipase A2, eIF4E-eukaryotic translation initiation factor 4E).

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Substrate</th>
<th>Sequence</th>
<th>Phos. site</th>
<th>monocytes</th>
<th>Monocytes</th>
<th>MDM</th>
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<tbody>
<tr>
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<td></td>
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<tr>
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DISCUSSION
In the present study it was shown that monocytes respond better to p38 MAPK inhibition than monocyte-derived macrophages, and also that cell differentiation is influenced by p38 MAPK inhibition. The importance of synovial macrophages in inflammation has been established in several studies, and recently it has been reported that the number of synovial sublining macrophages does correlate well with clinical improvement after antirheumatic treatment (MTX, leflunomide, Infliximab, prednisone)\(^{15}\). In previous work from our group it has been shown that TNF-\(\alpha\) production by monocyte-derived macrophages was effectively inhibited by the p38 MAPK inhibitor RWJ 67657\(^{10}\). As p38 MAPK inhibitors are in phase II clinical trials in RA\(^{16}\) knowledge of the exact effects of such drugs is important.

TNF-\(\alpha\) production was higher and more effectively inhibited by RWJ 67657 (93%) in monocytes than in MDM (45%), whereas IL-1\(\beta\) production was inhibited in monocytes (93%), but was not excreted by MDM. This latter finding is in concordance with the study by Herzyk \textit{et al.}\(^{17}\) and is related to the difficulty of release of IL-1\(\beta\) by macrophages. IL-8 production was tenfold higher in monocytes, but effects of p38 MAPK inhibitor treatment were comparable. In contrast to cytokine production was MMP-9 abundantly produced by MDM, even in unstimulated cells. Inhibition of MMP-9 by p38 MAPK inhibitor treatment in monocytes was only 21%, and in MDM 37%, indicating that pathways other than the p38 MAPK pathway must be involved in MMP-9 regulation. Lai \textit{et al.}\(^{18}\) demonstrated a dominant role for the ERK1/2 pathway in MMP-9 regulation in human monocytes.

With real-time RT-PCR we found higher expression of TNF-\(\alpha\), IL-1\(\beta\) and IL-8 mRNA levels in MDM compared to monocytes. Although protein production in monocytes was higher, mRNA levels seem to accumulate in MDM. It has been known that activation of p38 MAPK has a stabilizing effect on mRNA of inflammatory genes. This effect has been described for TNF-\(\alpha\) both for primary human monocytes\(^{19}\) as well as for macrophage-like cell lines\(^{20}\). The results from our study show that reduction of mRNA expression of TNF-\(\alpha\) and IL-1\(\beta\) by p38 MAPK inhibition was approximately the same for monocytes and MDM (81% and 79% (TNF-\(\alpha\)), and 75% and 68% (IL-1\(\beta\)) respectively), whereas IL-8 and MMP-9 mRNA was more reduced in monocytes than MDM due to RWJ 67657 treatment (77% and 36% (IL-8), 42% and 11% (MMP-9) respectively).

From the differentiation experiments it could be demonstrated that already after one day of differerentiation with M-CSF, the properties of the monocytes changed: IL-1\(\beta\) was no longer produced, TNF-\(\alpha\) production was very low, whereas MMP-9 production increased. Differentiation in the presence of RWJ 67657 significantly reduced TNF-\(\alpha\) protein and mRNA expression and IL-8 and MMP-9 protein production. Ayala \textit{et al.}\(^{21}\) reported that p38 MAPK inhibition at concentrations, which were reported to inhibit MAPKAPK-2 activation, blocked monocyte differentiation and chemotaxis. From our data we can not conclude that differentiation is blocked, but rather that protein production is decreased due to the p38 MAPK inhibition.

The results from the kinase array showed that indeed the substrates involved in the p38 MAPK pathway were phosphorylated by activation both in monocytes and in MDM. From the literature it is known that \textit{in vitro} MKK3, MKK4, and MKK6 all show a
strong preference for phosphorylation of the tyrosine residue of the Thr-Gly-Tyr motif in the p38 MAPK substrate \(^{21}\) and this is in concordance with our findings. Recently it was reported that oxidative stress of resident vascular cells and macrophages potently enhances eIF4E phosphorylation by the activation of Mnk-1 \(^{22}\). Phosphorylation of eukaryotic translation initiation factor 4E is associated with increased activity of the translational machinery. With this array the activation status of several kinases in a single experiment can be investigated, but more research is needed to understand the interactions of the intracellular signal transduction pathways.

REFERENCES

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