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The influence of toll-like receptor (TLR-) agonists on lysozyme activity, TNF-alpha secretion and intercellular adhesion in THP-1 cells

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Monocytes play a major role in modulating inflammation and can be found as circulating blood cells or, after extravasation, as differentiated tissue specific macrophages. The activation of TLRs (Toll-like receptors) modulates their action and contributes considerably to the eradication of pathogens. Aim of this study was to investigate the influence of TLR-agonists on TNF-alpha secretion and lysozyme activity in a comparative assay. To explore the importance of differentiation the monocytic cell line THP-1 was pre-treated with PMA and IFNy. Stimulation of undifferentiated cells with various bacterial antigens had almost no effect on TNF-alpha secretion and lysozyme activity but influenced adhesion molecule expression clearly. Differentiated THP-1 cells were much more sensitive to further stimulation with TLR-agonists yet revealed an opposing effect on lysozyme activity and TNF-alpha secretion. The results suggest a coordinated activation of intercellular adhesion molecule expression and cytokine secretion in the differentiation process from monocytes to macrophages.

1. Introduction

The differentiation of hematopoietic stem cells into mature immunocompetent cells depends on the balanced expression and secretion of numerous different proteins including transcription factors (TFs) and membrane receptors as well as cytokines and growth factors (Martinez et al. 2006; Valledor et al. 1998). Two major hematopoietic precursor cell lineages can be differentiated – common lymphoid progenitors and common myeloid progenitors, with the first giving rise to B-lymphocytes, T-lymphocytes and natural killer (NK) cell lineages and the latter differentiating into the second subset of immune cells including neutrophils, eosinophils, basophils, mast cells, megakaryocytes, erythrocytes, monocytes and macrophages (Chaplin 2010).
Leukocyte rolling, adhesion and transmigration were described a long time ago but researchers only recently began to unravel the underlying molecular mechanisms. A coordinated cascade of selectin-mediated rolling, chemokine-triggered activation, integrin-dependent arrest and subsequent transendothelial migration is not only important for replenishing tissue macrophages and dendritic cells but also for monocytes to enter sites of infection in order to eradicate invading pathogens (Ley et al. 2007). Once activated, macrophages show a strong phagocytic potential and antimicrobial activity (Chaplin 2010). As part of the innate immune system, they produce high amounts of bacteriolytic lysozyme which can be found in various body fluids and organs (Klockars and Reitamo 1975). Lysozyme is a small cationic glycosidase that catalyzes the hydrolysis of the linkage between N-acetylmuramic acid and N-acetyl glucosamine, a glycosidic linkage found in the cell walls of Gram-positive bacteria (Kirby 2001). Although activated macrophages display pro-inflammatory activity their implication as immune modulators with pro- and anti-inflammatory properties becomes more and more evident (Benoit et al. 2008; Mosser 2003). In addition to their phagocytic potential and the secretion of reactive oxygen or nitrogen species (Forman and Torres 2001), macrophages act as immunologic regulators according to their receptor expression and cytokine secretion profile (Gordon 2003; Martinez et al. 2006).
Pattern recognition receptors mediate the immunological response to bacterial infections. Among them, a family of evolutionally well conserved cell-surface and intracellular receptors – the Toll-like receptor (TLR)-family, has shown its relevance in proper immune function (Kawai and Akira 2010). The activation of TLRs occurs through the binding of certain bacterial components like lipopeptides, lipopolysaccharides (LPS) or polynucleotides and results *inter alia* in the activat6ion of the master TF Nuclear Factor κB (NF-κB).
Tumor necrosis factor alpha (TNF-alpha) is a key pro-inflammatory cytokine that is implicated in various inflammatory diseases and important in the response to infection (Bradley 2008). Since not only the stimulation with TLR-agonists like LPS leads to an elevated TNF-alpha expression and secretion but that using anti-TNF-alpha drugs in a variety of inflammatory conditions leads to an increased risk of bacterial infections further supports the important physiological role of TNF-alpha

Abbreviations: C/EBPβ, CCAAT/enhancer binding protein β; ICAM-1, intercellular adhesion molecule-1; IRF, interferon regulatory factor; LPS, lipopolysaccharides; NF- κB, nuclear factor kappa B; PMA, phorbol 12-myristate 13-acetate; PSGL-1, P-selectin glycoprotein ligand-1; ESL-1, E-selectin ligand-1; Mac-1, macrophage receptor 1; STAT, signal transducer and activator of transcription; SOCS, suppressor of cytokine activation; TF, transcription factor; TLR, Toll-like receptor; VCAM-1, vascular adhesion molecule-1.

in infection (Raychaudhuri et al. 2009; Takashiba et al. 1999; Yeganeh et al. 2008). The expression of TNF-alpha is influenced by NF- κB and CCAAT/enhancer binding protein β (C/EBPβ), two TFs that also stimulate lysozyme expression in mammals (Lefevre et al. 2001, 2003; Liu et al. 2000; Phi van 1996; Pope et al. 2000).

Since both, NF- κB and C/EBPβ are important down-stream effector proteins in the TLR-mediated (TLR4/MyD88-dependent) increase in TNF-alpha secretion (Lu et al. 2009) an implication of TLRs in lysozyme secretion seemed obvious. Interestingly, previous studies investigating the role of lysozyme considered mainly its regulation on a chromatin- and/or mRNA-level, especially in chicken cell lines, yet little is known about the actual lysozyme secretion under inflammatory conditions in a human cell system. Moreover, most of the studies analyzing the influence of TLRs on TNF-alpha secretion and lysozyme expression in monocytes and macrophages were conducted with the TLR2/4-agonist LPS, whereas information about the role of other TLR-agonists in this context is scarce.

To elucidate the influence of TLR-activation on TNF-alpha and lysozyme secretion, ten different TLR-agonists were analyzed in a comparative assay in the human monocytic cell line THP-1 under differentiated and undifferentiated conditions. Furthermore, the influence of TLR-activation in the context of intercellular adhesion and adhesion molecule expression was investigated and the results are presented in this paper.

2. Investigations and results

2.1. Influence of PMA, IFNy and LPS on lysozyme activity and TNF-alpha secretion

THP-1 cells grow in suspension with little cluster formation and can be differentiated with the phorbol diester phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C (PKC) which does not only alter the cells phenotypic appearance but also primes them for stimulation with LPS (Greenberger et al. 1980; Takashiba et al. 1999).

TNF-alpha is known to be mainly produced by activated macrophages and T-Lymphocytes and to act as a key regulator in inflammatory responses (Bradley 2008), yet the effect of PMA, as a differentiation stimulating agent, on the secretion of TNF-alpha in THP-1 cells seems controversial (Schwende et al. 1996; Takashiba et al. 1999). To establish a stable model for the differentiation THP-1 cells were stimulated with PMA-concentrations ranging from 10ng/mL to 1000 ng/mL for 72 h and the supernatants were submitted to analysis. Determined by ELISA, the differentiation with PMA resulted in a significant increase of secreted TNF-alpha in a dose dependent manner (data not shown). In the context of lysozyme secretion, only the stimulation with a concentration of 10 ng/mL PMA resulted in an almost 4-fold increase in lysozyme activity whereas higher concentrations led to a much lower increase of less than 2-fold (data not shown).Although alternative differentiation protocols had been suggested (Daigneault et al. 2010; Park et al. 2007), stable differentiation for more than 72 h could only be achieved with PMA concentrations higher than 100 ng/mL (Fig. 1a, b). For this reason, a final concentration of 100 ng/mL PMA was used to differentiate the monocytic cell line THP-1 into macrophages for further experiments in this study.These results confirm that PMA can not only induce differentiation in THP-1 cells but also increase TNF-alpha secretion in THP-1 cells in a dose dependent manner.

To investigate the influence of PMA and IFNy on the responsiveness to stimulation with the TLR4 agonist LPS THP-1 cells were differentiated with PMA and treated with IFNy prior to stimulation with LPS or left untreated. Undifferentiated THP-1 cells responded only slightly with an increase in TNF-alpha secretion and lysozyme activity under treatment with IFNy whereas LPS seemed to be ineffective (Fig. 1c, e). In contrast, PMA-differentiated THP-1 cells were much more sensitive to both stimulation with IFNy and LPS. The stimulation with IFNy resulted in a 3-fold increase in TNF-alpha secretion. This stimulatory effect was further enhanced by additional LPS treatmentfor both IFNy pre-treated and untreated THP-1 cells (Fig. 1d). In the context of lysozyme, pre-treatment with IFNy resulted in an almost 4-fold increase in measurable activity. Interestingly further addition of LPS into the medium reduced lysozyme activity. This inhibitory effect of LPS was detectable for both IFNy treated and untreated THP-1 cells (Fig. 1f).

2.2. Effect of TLR-agonists on TNF-alpha secretion and lysozyme activity

To investigate the influence of IFNy and bacterial cell components like lipopeptides and oligonucleotides on the secretion of TNF-alpha and lysozyme activity differentiated and undifferentiated THP-1 cells were stimulated with various TLR-agonists for 24 h in a comparative assay. The stimulation of undifferentiated THP-1 cells with IFNy had no clear effect on the amount of secreted TNF-alpha (Fig. 2a). But stimulation with the TLR-agonists Pam3CSK4, HKLM and Poly(I:C) resulted in a 1.5-fold increase compared to control. Only the stimulation with the TLR-agonist FSL-1led to a 2- to 3-fold increase in secreted TNF-alpha both in IFNy pre-treated and untreated THP-1 cells (Fig. 2a). In contrast, differentiated THP-1 cells responded with a distinct response to the stimulation with IFNy as well as the stimulation with TLR-agonists (Fig. 2b). Pre-treatment of differentiated THP-1 cells with IFNy resulted in an almost 10-fold increase of detectable TNF-alpha in the medium. The response to stimulation with the various TLR-agonists was similar both in IFNy pre-treated and untreated THP-1 cells yet only TLG-agonists that targeted cell-surface TLRs were effective (Fig. 2b), namely Pam3CSK4, HKLM, ST-FLA, FSL-1 and LPS (for LPS see Fig. 1d). As in undifferentiated cells, stimulation with the TLR-agonist FSL-1 had the strongest effect both, in IFNy untreated as well as in IFNy pre-treated differentiated THP-1 cells (Fig 2a, b). Although the overall TNF-alpha secretion was elevated in the presence of IFNy the relative influence of the different TLR-agonists was not so distinct in differentiated, IFNy pre-treated cells when compared to the relative influence in differentiated, IFNy untreated cells (Fig. 2b).

Since lysozyme targets the linkage between N-acetylmuramic acid and N-acetyl glucosamine, which leads to the degradation of certain types of bacteria, the question arouse whether the stimulation of monocytes and/or macrophages with different TLR-agonists would influence the secretion of active lysozyme. To investigate the influence of a putative priming effect of IFNy, both, differentiated and undifferentiated THP-1 cells were pre-stimulated with IFNy or PBS, respectively (Fig. 2c, d). Undifferentiated THP-1 cells showed only little responsiveness to IFNy pre-treatment and the stimulation with various TLR-agonists had no significant effect (Fig. 2c). In contrast, differentiated THP-1 cells responded with a 2.5 fold increase in lysozyme activity to stimulation with IFNy. And, although the effect was small, TLR-agonists that targeted cell-surface TLRs led to a slight decrease in lysozyme activity whereas intracellular TLR-agonists had no effect at all (Fig. 2d).

2.3. Changes in RNA-expression in response to various TLR-agonists

The rolling of leukocytes on vascular surfaces facilitates their migration into sites of infection and inflammation and requires

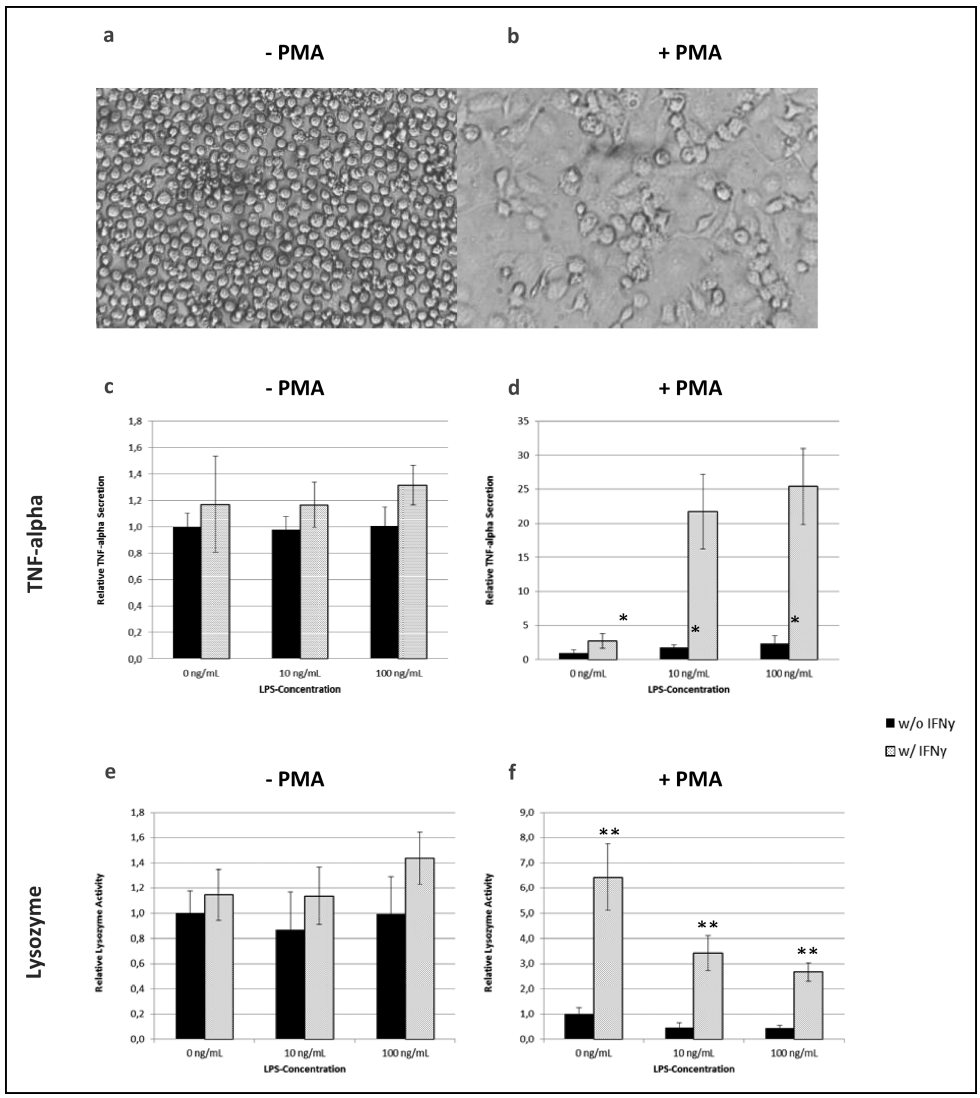

Fig. 1: Influence of PMA, IFNy and LPS on TNF-alpha secretion and lysozyme activity in THP-1 cells. THP-1 cells were seeded at a density of 500.000 cells/mL (250.000 cells per well) in a 24-well plate. Immediately after seeding, cells were differentiated with PMA (100 ng/mL) or treated with DMSO (1:10000) as control. 48 h after differentiation, a medium change with reduced levels of FCS (RPMI + 1% FCS + 1% L-Glutamine) was conducted. Immediately after seeding, undifferentiated (-PMA) and differentiated (+PMA) THP-1 cells were stimulated with LPS in various concentrations for 24 h in the presence and absence of IFNy. **a** and **b**: THP-1 cells were stimulated with PMA (100 ng/mL) for three days. Pictures were taken 72 h after seeding at a magnification of 100 x.**c** and **d:** TNF-alpha secretion was determined by ELISA. **e** and **f:** Lysozyme activity was measured enzymatically. **c – f:** Bars represent normalized mean values + /- SD of three individual experiments. Each experiment was conducted in triplicates. Asterix indicate significant difference to control (0 ng/mL LPS; w/o IFNy *, w/ IFNy **), $\alpha < 0.05$ (U-test).

a coordinated expression of cell-surface receptors. Adhesion molecules like P-selectin glycoprotein ligand 1 (PSGL-1), E-selectin ligand 1 (ESL-1), L-selectin and macrophage receptor 1 (MAC-1) are involved in monocyte trafficking and coordinate the process of extravasation from initial adhesion and rolling to transmigration into the tissue (Hidalgo et al. 2007; Ley et al. 2007; McEver and Zhu 2010; Shi and Pamer 2011). Previous studies have shown that TLR-agonists are able to induce adhesion and migration of the macrophage-like cell line RAW264.7 and various human colon cancer cells via induction of NF-κB and ROS (Maa et al. 2011; O'Leary et al. 2012). Interestingly, the stimulation of THP-1 cells with IFNy and various TLR-agonists induced a strong aggregation of cells into clusters which indicates a putative involvement of bacterial antigens in the expression of intercellular adhesion molecules. Since the differentiation of THP-1 cells induced a strong adherence to the bottom of the plates no further changes in morphology in response to stimulation with TLR-agonists and/or IFNy could be distinguished (Fig. 1b). Undifferentiated cells, however, behaved differently. IFNy untreated THP-1 cells showed almost no morphological alteration in response to TLR-agonists whereas IFNy pre-treated cells responded with the formation of clusters in varying intensities (Fig. 3). Although IFNy alone induced a slight intercellular adhesion (Fig. 3b) further addition of TLR-agonists targeting cell-surface TLRs caused an even stronger aggregation (Fig. 3c, d) compared to TLR-agonists that stimulate intracellular TLRs (data not shown) where no adhesion was visible.

To assess whether the presence of TLR-agonists has an influenceon the extravasation process of circulating monocytes by changingthe expression levels of cell surface receptors involved in rolling and adhesion RNA-expression levels were analyzed using RT-qPCR. Interestingly, although undifferentiated THP-1 cells did not respond with an increase in TNF-alpha secretion they however respond with a significant augmentation of TNF-alpha RNA expression (compare Fig. 1c and 4). Of the cell adhesion molecules investigated in this study only MAC-1, which encodes the integrin alpha M chain, did not respond to stimulation with either IFNy or LPS or both together. In contrast, PSGL-1 and CD86 RNA-expression was clearly induced upon treatment with IFNy and LPS. Yet in both cases the response to IFNy was stronger compared to that of LPS, ranging from 1.5–3 fold for LPS and 3–4 fold for IFNy. Stimulation with both, IFNy and LPS,together caused, although not significantly,

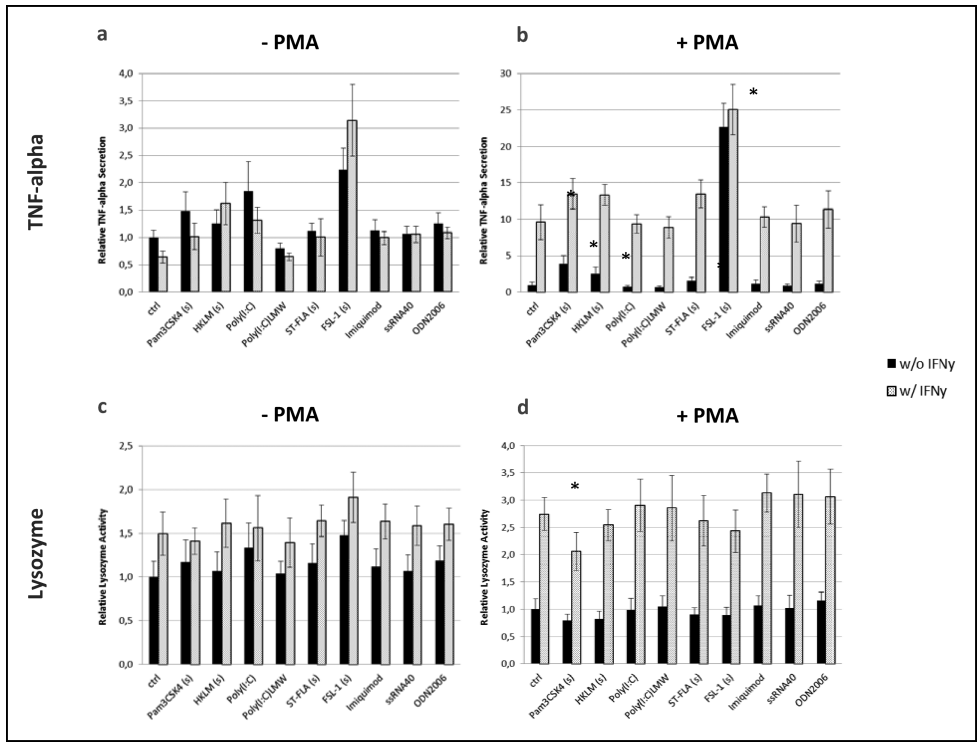

Fig. 2: Influence of IFNy and TLR-agonists on TNF-alpha secretion and lysozyme activity in undifferentiated and differentiated THP-1 cells. THP-1 cells were differentiated with PMA (100 ng/mL) for 48 h. Undifferentiated cells were treated with DMSO (1:10000) as control. Priming of both undifferentiated and differentiated cells was performed with IFNy (10 ng/mL) or PBS 24 h after seeding. Stimulation with TLR-agonists (100 ng/mL) as well as second IFNy-priming occurred after a medium change with serum-reduced RPMI 48 h after seeding. **a** and **b** - Secreted TNF-alpha was quantified by ELISA. **c** and **d** - Active lysozyme was quantified using an enzymatic activity assay. Bars represent mean values + /- SD of four individual experiments. Each experiment was conducted in triplicates. S in brackets indicates TLR-agonists targeting cell-surface receptors. * Significant difference to control (0 ng/mL LPS; w/o IFNy), $a < 0.05$ (U-test).

the strongest induction of PSGL-1 and CD86 RNA-expression. L-Selectin did only response to IFNy and not to stimulation with LPS. ESL-1 showed only little responsiveness to stimulation with either IFNy or LPS, whereas a simultaneous treatment with both substances almost doubled the amount of expressed RNA (Fig. 4).

3. Discussion

Differentiation of monocytes into macrophages is an important step for proper pathogen eradication and immune regulation in the tissue. The results in this study show that in undifferentiated THP-1 cells the TNF-alpha secretion and lysozyme activity are barely influenced by IFNy as well as LPS whereas the differ-

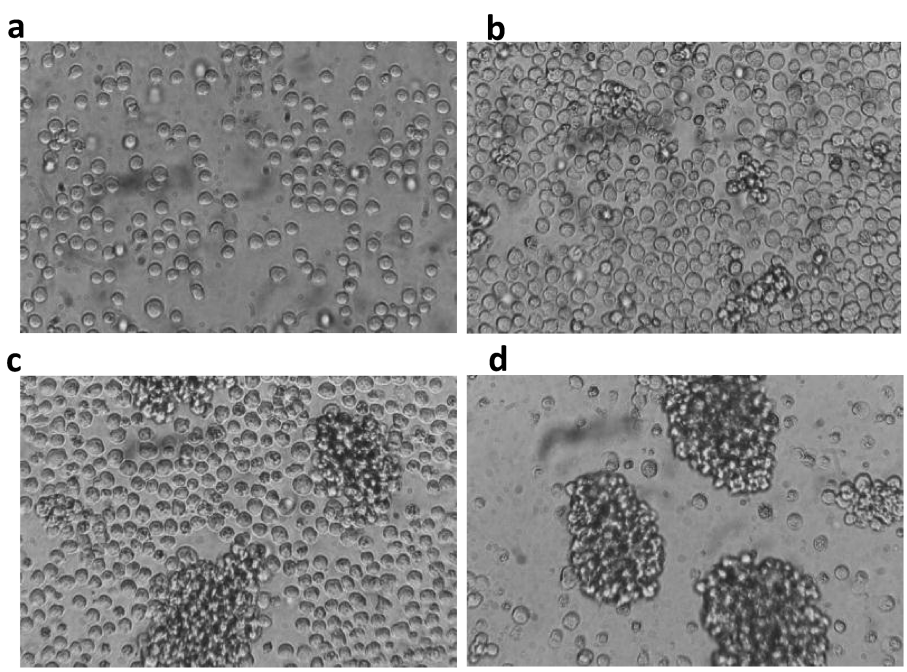

Fig. 3: Morphological changes due to stimulation with various TLR-agonists. Undifferentiated THP-1 cells were treated with DMSO (1:10000) for 48 h as control. Priming was performed with IFNy (10 ng/mL) or PBS 24 h after seeding. Stimulation with TLR-agonists (100 ng/mL) as well as second IFNy-priming occurred after a medium change with serum-reduced RPMI 48 h after seeding. Pictures were taken 24 h after stimulation with TLR-agonists. a – THP-1 cells at 0 h; b-d: after 72 h: b – (- PMA; + IFNy; + H_2O); c – (- PMA; + IFNy; + LPS); d – (- PMA; + IFNy; + FSL-1).

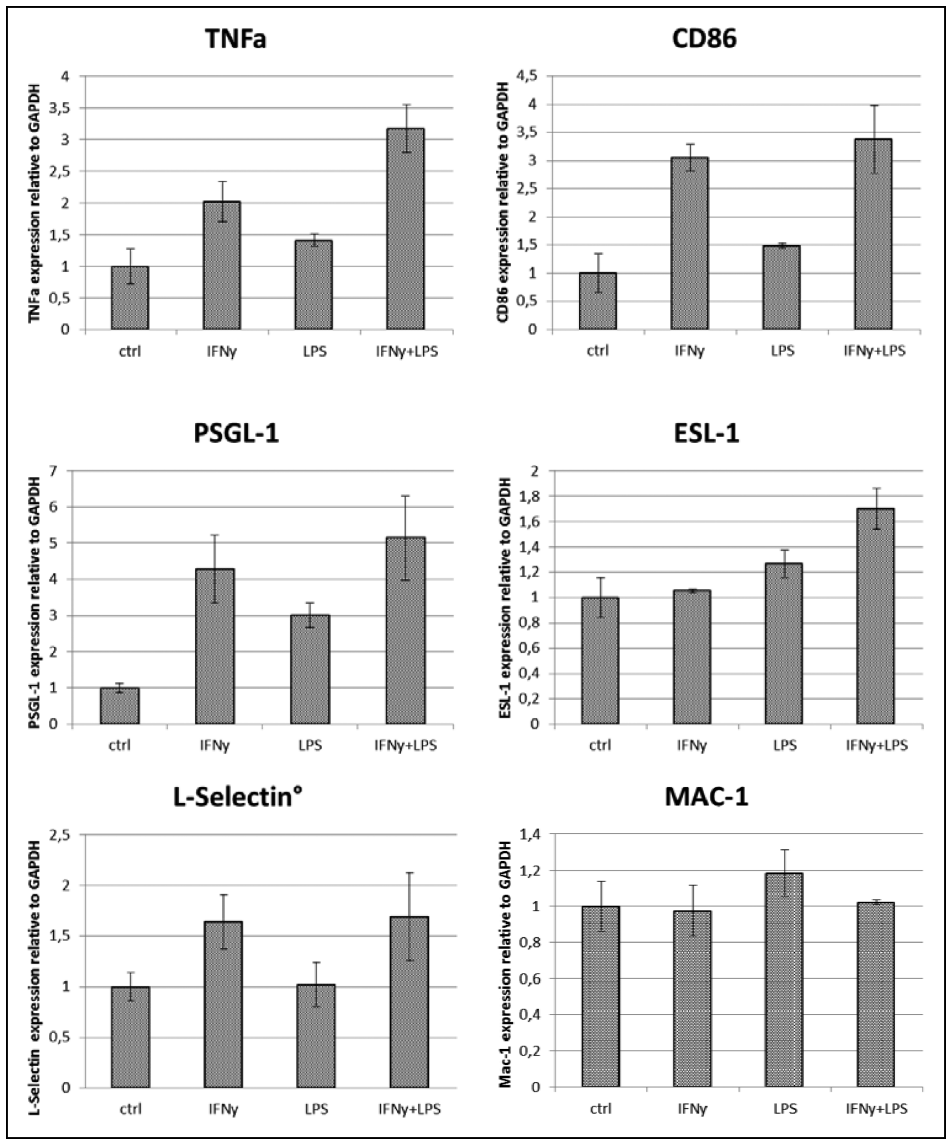

Fig. 4: Influence of IFNy and LPS on RNA-expression of receptors involved in adhesion and rolling. THP-1 cells were seeded at a density 500.000 cells/mL in a 24-well plate and incubated for 24 h at 37 °C. After 24 h cells were stimulated with IFNy and LPS for further 24 h until aggregation of cells into clusters was clearly visible. At day three cells were harvested submitted to RNA-Analysis. Bars show representative mean values + /- SDof three individual experiments each performed in triplicates. ° Bars show mean values + /- SDof all three individual experiments to show representative values.

entiation with PMA sensitizes them for the influence of LPS which has been described previously (Pradines-Figueres and Raetz 1992; Regenhard et al. 2001). Our data show further that PMA and hence the activation of PKC also sensitizes THP-1 cells for the stimulation with IFNy which, in differentiated THP-1 cells, acts positively on both lysozyme activity and TNF-alpha secretion.

The role of LPS and other TLR-agonists in THP-1 cells seems puzzling. Like TNF-alpha, lysozyme plays an important role in the host defense against invading pathogens and the expression of both is positively influenced by TFs like NF-κB and C/EBPβ (Goethe and Phi-van 1997; Lefevre et al. 2001; Lefevre et al. 2003; Liu et al. 2000; Lu et al. 2009; Phi van 1996; Pope et al. 2000). Yet, on the basis of the results obtained in this study, the stimulation with the TLR-agonists Pam3CSK4, HKLM, LPS-EK, ST-FLA and FSL-1 has opposing effects on lysozyme activity and TNF-alpha secretion in THP-1 cells, with a stimulation of TNF-alpha secretion on the one hand and an inhibition of lysozyme activity on the other. It is interesting that both effector proteins, TNF-alpha and lysozyme, are regulated by similar TFs and both participate in the eradication of invading pathogens yet their regulation in the presence of pathogens seems to be regulated differently and, at least partly, by antagonizing mechanisms.

The stimulation with LPS results in an increase in lysozyme expression and has been described previously but post transcriptional regulatory mechanisms have only poorly been taken into account. Goethe and Phi-van (1998) investigated the influence of LPS on the regulation of the primary transcript of the lysozyme gene in HD11 cells and found that LPS stimulation led to an increase in lysozyme pre-mRNAs as well as to changes in the poly(A) tail length. Although polysomal recruitment was not affected by changes in poly(A) tail length and the levels of mature lysozyme mRNA in the cytoplasm remained constant during the incubation time periods the authors found an increase in intracellular and secreted lysozyme protein levels which appears quite confusing. Yet the results in our study show a clear reduction in lysozyme activity in the medium upon LPS stimulation. One of the major differences between the experimental procedures was the priming with IFNy. Among other proteins that promote microbe destruction in macrophages (Schroder et al. 2004) IFNy is a potent macrophage activator that, especially at low concentrations, primes these cells for the stimulation with endotoxins like LPS and CpG-DNA

(Lorsbach et al. 1993; Sweet et al. 1998; Williams et al. 1992). This pro-inflammatory feed-forward loop is mediated by the accumulation of intracellular signal-transducer and activator of transcription 1 (STAT1) and its cooperation with NF-κB in the activation of certain gene promotors. Since the expression of lysozyme and TNF-alpha is influenced positively by NF-κB it was not surprising that stimulation with IFNy resulted in an increase in actual protein secretion of both proteins. Higher concentrations of IFNy, in contrast, rather act inhibitory *via* the activation of SOCS1 (suppressor of cytokine activation) which antagonizes the pro-inflammatory actions of STAT1 by (i) competing for the same docking sites on the IFNGR and (ii) by decreasing NF-κB stability in the nucleus (Hu et al. 2008; Strebovsky et al. 2011). This STAT1 mediated SOCS1 activation acts *via* an increased expression of IRF1 (interferon regulatory factor 1), a positive regulator of SOCS1 transcription in human keratinocytes which is also implicated in LPS-mediated signaling (Madonna et al. 2010; Nhu et al. 2006). Although both stimulation with IFNy and LPS cumulate in the activation of NF-κB they also induce inhibitory feedback loops that prevent exceeded inflammation. Activation of TLR4 is known to signal *via* two intracellular pathways: The MYD88-dependent and MYD88-independent pathway to activate pro-inflammatory cytokines. The MYD88-dependent pathway results in the activation of NF-κB and its translocation into the nucleus whereas the MYD88-independent pathway acts *via* IRF3 which maintains the expression of pro-inflammatory mediators like TNF-alpha or IFNß independent of MYD88 and hence the activation of NF-κB (Mogensen 2009; Moynagh 2005). This circumstance might account for the maintenance of TNF-alpha secretion even though feedback inhibitory loops are active. It seems that lysozyme expression is induced by both IFNy and LPS alone whereas a simultaneous activation favors inhibitory feedback loops and results in a decrease in lysozyme secretion. Further investigation is necessary to elucidate the factors that become activated in response to TLR-stimulation and how they participate in lysozyme activity and TNF-alpha secretion as well. Since TLR-activation not only activates lysozyme expression but also leads to post transcriptional modifications (Goethe and Phi-van 1998) other mechanisms like processing and secretion must be taken into account.

A comparison of Figs. 2 and 3 shows an interesting correlation between TNF-secretion and the phenotype of undifferentiated THP-1 cells. The TLR-agonists that induced TNF-alpha secretion in differentiated cells also caused the formation of clusters in undifferentiated THP-1 cells. The activation of TLRs is known to induce many proteins including cytokines and adhesion molecules which are important for the migration of monocytes into lymphoid organs or inflamed tissues (Imhof and Aurrand-Lions 2004; Newton and Dixit 2012). Although undifferentiated THP-1 cells failed to induce TNF-alpha secretion and lysozyme activity in response to stimulation with the TLR-agonists Pam3CSK4, HKLM, LPS-EK and ST-FLA, their intercellular adhesion ability was enhanced significantly (Figs. 2 and 3, data not shown). Especially the TLR6/2-agonist FSL-1, which also induced TNF-alpha secretion independent of PMA and IFNy treatment, induced a strong cell agglomeration and indicates a particular importance of TLR6 in the initial activation of circulating monocytes. TLR6 has been shown to be expressed on the surface of various leukocytes including THP-1 cells (Nakao et al. 2005) and its activation by FSL-1 causes the expression of cell surface molecules like ICAM1 (intercellular adhesion molecule-1) on human gingival fibroblasts (Shibata et al. 2000) as well as proinflammatory chemokines in THP-1 cells (Won et al. 2012). According to the data presented in this paper TLR6/2 seems to play an important role for the defense against invading pathogens since its activation not only leads to a strong TNF-alpha secretion but to an increased cell-adhesion as well. Further experiments are necessary to find out which mechanisms are responsible forthe observed agglomeration of THP-1 cells by the TLR6/2 activator FSL-1.

RT-qPCR analysis revealed that the expression of the cell adhesion molecules PSGL-1 and CD86 is positively affected by both stimulation with IFNy and LPS. PSGL-1 is expressed by monocytes and can bind to P-selectin, E-selectin and L-selectin (Martinez et al. 2005). Interaction of PSGL-1 with P-selectin and E-selectin is a critical step for initiation of leucocyte rolling on endothelial surfaces. Together with ESL-1, which mainly interacts with E-selectin, both proteins allow binding of circulating leukocytes to the endothelium under blood flow conditions. Interaction of PSGL-1 with L-selectin, which is expressed by most leukocytes, promotes tethering of leukocytes with each other and recruits more circulating monocytes to inflamed tissue (Ley et al. 2007; Martinez et al. 2005). TNF-alpha and LPS synergistically activate endothelial expression of intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) (Jersmann et al. 2001). As demonstrated by the data presented in this paper TLR-agonists like LPS are also able to induce adhesion molecule expression in monocytes. It appears likely that TLR-agonists act as attractants that prime endothelial cells nearby infected tissue as well as circulating monocytes and thus facilitate rolling and adhesion in proximity to infected tissue. According to the microscopical data this effect seems not only to be true for the activation of TLR4 but other TLRs, especially cell surface-receptors as well. Once monocytes have entered sites of infection they become further activated by inflammatory stimuli that lead inter alia to activation of PKC. Together with IFNy and LPS, the activation of PKC strongly induces TNF-alpha secretion which further supports monocyte recruitment to infected tissue by inducing adhesion molecule expression in endothelial cells (Fig. 5).

4. Experimental

4.1. Cell culture

Certified THP-1 cells (DSMZ, Braunschweig, Germany) were maintained in RPMI Medium without phenol red supplemented with 10 % FCS and 1% L-glutamine (all from Biochrom AG, Germany) at 37 °C and 5 % CO_2. Passaging of cells occurred twice a week at a ratio of 1:4 and 1:3, respectively. To avoid age-related genomic alterations, cells were kept in culture for no longer than twenty passages. Since the cultivation of cell lines with antimicrobials leads to changes in protein expression (Mathieson et al. 2011) the THP-1 cells used in this work were cultivated in the absence of any antibiotic or antifungal agent.

4.2. Differentiation and stimulation of THP-1 cells

Although many differentiation protocols have been described previously the role of IFNy in this context has only poorly been taken into account. In our model THP-1 cells were seeded in 24-well plates at a density of 500.000 cells/mL (250.000 cells per well) in RPMI medium supplemented with 10 % fetal calf serum (FCS) and 1 % L-glutamine. Immediately after seeding (0 h), cells were treated with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Germany) at a final concentration of 100 ng/mL for 48 h. PMA was previously dissolved in dimethyl sulfoxide (DMSO) and diluted with GIBCO water for injection (Life Technologies, USA) to a working concentration of 10 µg/mL. As control, undifferentiated cells were treated with DMSO for 48 h at a corresponding ratio of 1:10000. IFNy (Cell Signaling Technologies, USA) was diluted in PBS (Biochrome AG, Germany) to a working concentration of 500 ng/mL and the stimulation occurred 24 h after seeding at a final concentration of 10 ng/mL. Treatment with PBS served as control. 48 h after seeding, a medium change was carried out with RPMI supplemented with only 1 % FCS and 1 % L-glutamine. Immediately after medium change, IFNy-stimulation was repeated and the cells were stimulated with different TLR-agonists dissolved in GIBCO water for incjection at a final concentration of 100 ng/mL. Treatment with PBS and water, respectively, served as control. The following TLR-agonists were used in this study (all provided by Invivogen, USA): Pam3CSK4 (TLR1/2 agonist), HKLM (TLR2 agonist), Poly (I:C) and Poly (I:C) LMW (TLR3

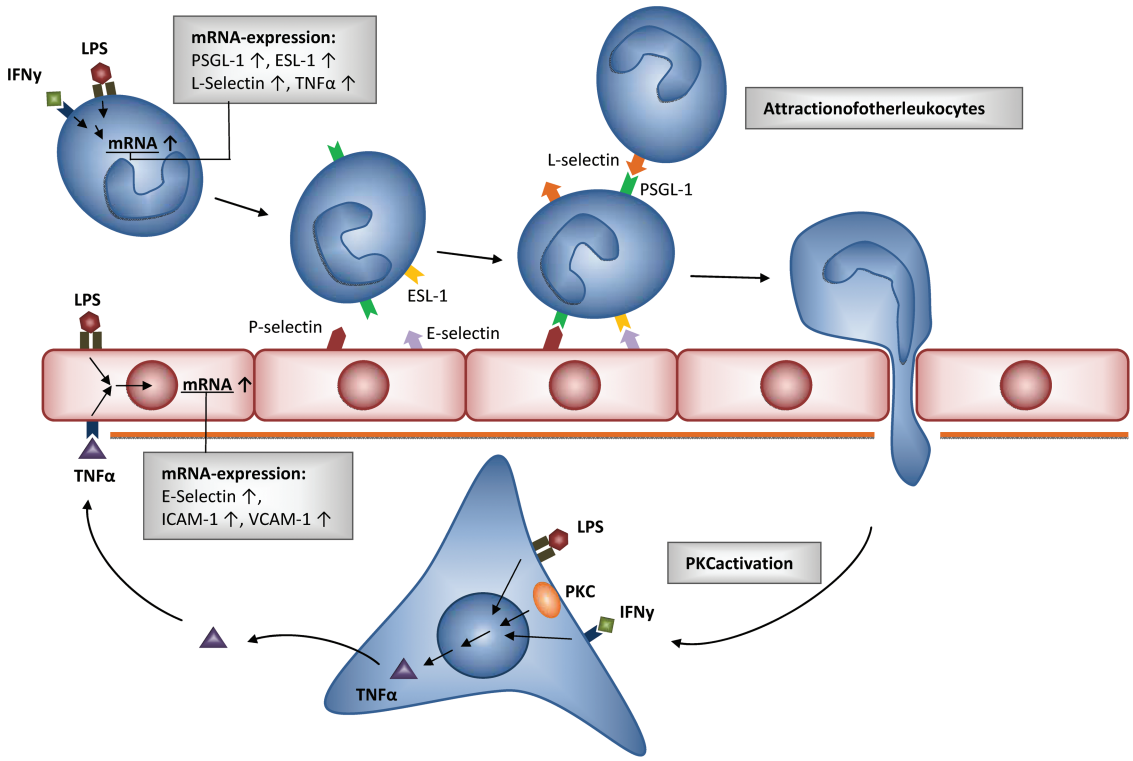

Fig. 5: Proposed model for monocyte recruitment and macrophage activation. Circulating monocytes that get in contact with TLR-agonists and IFNy get primed for extravasation by expressing cell adhesion molecules important in endothelial rolling and adhesion. Endothelial cells in proximity to infected tissue get activated by LPS and TNF-alpha which leads to expression of cell-surface receptors like selectins, ICAM-1 and VCAM-1 that in turn serve as anchor proteins for activated circulating monocytes. Once monocytes have entered inflamed tissue and acquire macrophage like properties they further stimulate this recruitment process by secreting pro-inflammatory cytokines like e.g. TNF-alpha.

agonist), LPS (TLR4 agonist), ST-FLA (Flagellin, TLR5 agonist), FSL-1 (TLR6/2 agonist), Imiquimod (TLR7 agonist), ssRNA40 (TLR8 agonist) and ODN2006 (TLR9 agonist. For RNA-quantification analysis, undifferentiated THP-1 cells were seeded at a density 500.000 cells/mL in a 24-well plate and incubated for 24 h at 37 °C and 5 % CO_2. Subsequently, cells were stimulated with IFNy and LPS at the above mentioned concentrations for further 24 h until aggregation of cells into clusters was clearly visible.

4.3. DNA-Quantification

Twenty-four hours after stimulation with TLR-agonists, supernatants of undifferentiated and differentiated cells were collected for further analysis. Undifferentiated cells were centrifuged and the cell pellet was resuspended in 1x DNA buffer (50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 2 M NaCl, 2 mM EDTA, pH 7,4). Adherent growing differentiated cells were covered with 1x DNA buffer. Disintegration of cells was performed by submission to repetitive freeze-and-thaw cycles. For quantification, 50 µL of the disintegrated cell suspension was combined with 50 µL HOECHST-solution (2 µg/mL HOECHST-33258 in 1x DNA buffer) and analyzed spectrophotometrically.

4.4. Lysozyme activity and TNF-alpha ELISA

To measure activity of secreted lysozyme, supernatants were collected 24 h after stimulation with TLR-agonists and analyzed with the EnzCheck® Lysozyme Assay Kit (Life Technologies, USA) according to the manufacturer's protocol. For quantification of secreted TNF-alpha, supernatants were collected 24 h after stimulation with TLR-Agonists and analyzed in an ELISA with the Human TNF-alpha total matched antibody pairs (eBioscience, USA) according to the manufacturer's protocol.

4.5. RT-qPCR analysis

Twenty-four hours after stimulation with IFNy and LPS RNA was extracted with the SeqLab RNA-Mini-Kit (SeqLab, Germany) according to the manufacturer's protocol. A DNase (Ambion, USA) digestion step with 1 Unit Enzyme for each column was performed in between steps 3 and 4. For cDNA-synthesis 1 µg of RNA (in 10 µL of nuclease free water [Life Technologies, USA]) were combined with 5 µL of Master Mix 1 (1 µL of 10 mM dNTP Mix and 1 µL of 0.5 µg/mL Oligo dT primer [both Qiagen, Germany], 4 µL nuclease free water) to allow primer annealing (5 min at 65 °C → 2 min at 4 °C). Subsequently, 10 µL of Master Mix 2 (0.7 µL MMLV reverse transcriptase and 5 µL 5 x First Strand Buffer [both Promega, USA], 0,5 µL Ribolock RNase Inhibitor [Fermentas, USA], 2 µL 0,1 M Dithiothreitol, 3 µL nuclease free water) were added and actual cDNA-synthesis initiated (2 h at 37 °C → 15 min at 75 °C → 4 °C). Quantification was performed on a Light Cycler 480 (Roche Applied Science, Germany) with the DyNAmo Flash Sybr Green qPCR Kit (Thermo Scientific, USA) according to the manufacturer's protocol.

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