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Sulphasalazine down-regulates the expression of the angiogenic factors platelet derived endothelial cell growth factor / thymidine phosphorylase and interleukin 8 in human monocytic-macrophage THP-1 and U937 cells

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Running title: Down-regulation of PD-ECGF/TP and IL-8 by sulphasalazine

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Abbreviations: PD-ECGF/TP, platelet derived endothelial cell growth factor / thymidine phosphorylase; IL-8, interleukin-8; NFκB, nuclear factor κB; SSZ, sulphasalazine

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Abstract

Platelet derived endothelial cell growth factor / thymidine phosphorylase (PD-ECGF/TP) and interleukin-8 (IL-8) are angiogenic factors produced by tumor infiltrating macrophages. Here we show that prolonged exposure of human monocytic/macrophage THP1 and U937 cells to sulphasalazine, an anti-inflammatory drug and inhibitor of NFκB, resulted in down-regulation of PD-ECGF/TP and IL-8 (mRNA, protein and activity) along with elimination of their induction by tumor necrosis factor-α and interferon-γ. Concomitantly, sulphasalazine-exposed cells were markedly resistant to 5'-deoxyfluorouridine, the last intermediate of capecitabine requiring activation by PD-ECGF/TP. This is the first report suggesting that disruption of NFκB dependent signaling pathways can provoke a marked and sustained down-regulation of macrophage-related angiogenic factors. However, this may also negatively affect capecitabine efficacy.

Introduction

Angiogenesis, the formation of new vasculature, plays a pivotal role in several pathological conditions including neoplastic and inflammatory diseases like rheumatoid arthritis (RA) (Folkman, 1995;Daly *et al.*, 2003). Angiogenesis is assumed to be regulated by the balance of pro- and anti-angiogenic factors produced by all the cell types involved. If the balance shifts towards the pro-angiogenic state, the formation of new vasculature is induced, as is the case in the majority of cancers and RA (Hanahan and Folkman, 1996). Infiltrating macrophages have been recognized as producers of either pro- or anti-angiogenic factors although the pro-pathological state usually prevails (Bingle *et al.*, 2002;Burmester *et al.*, 1997). Pro-angiogenic factors produced by macrophages include Interleukin-8 (IL-8) (Koch *et al.*, 1992) and platelet derived endothelial cell growth factor (PD-ECGF) (Takahashi *et al.*, 1996). Both factors were

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found to be upregulated in cervical cancer (Fujimoto *et al.*, 2002) and RA (Waguri *et al.*, 1997), suggesting a common co-regulatory pathway.

IL-8 is a so called CXC chemokine containing three amino acid residues Glu-Leu-Arg (ELR-motif) preceding the first conserved cysteine indicative of its pro-angiogenic action and is expressed in both cancer and RA (Belperio *et al.*, 2000). PD-ECGF is identical to the enzyme thymidine phosphorylase (TP; E.C. 2.4.2.4) and catalyzes the catabolism of thymidine (TdR) into thymine and deoxyribose-1-phosphate (Brown and Bicknell, 1998). In a cancer chemotherapeutic setting, TP is essential for the final activation of the 5-fluorouracil (5FU) pro-drug capecitabine by catalyzing the conversion of 5'-deoxyfluorouridine (5'DFUR) to 5FU (Ackland and Peters, 1999). PD-ECGF/TP is overexpressed in a variety of solid tumors (Miwa *et al.*, 1998) and is positively correlated with microvessel density (Toi *et al.*, 1995) and poor prognosis (Takebayashi *et al.*, 1996;Matsumura *et al.*, 1998). Similarly, elevated PD-ECGF/TP has been observed in serum and synovial fluids of RA patients (Waguri *et al.*, 1997).

It is well recognized that PD-ECGF/TP can be upregulated by cytokines such as tumor necrosis factor-α (TNF-α), IL-1 and interferon-γ (IFN-γ) (Eda *et al.*, 1993), various chemotherapeutic agents including taxanes, cyclophosphamide and mitomycin C (Sawada *et al.*, 1998), and X-ray radiation (Sawada *et al.*, 1999). The latter two may induce PD-ECGF/TP indirectly via up-regulation of TNF-α or IFN-γ (Blanquicett *et al.*, 2002).

Sulphasalazine (SSZ) is a slow acting anti-inflammatory drug commonly used in the second line treatment of RA and inflammatory bowel disease (Brooks, 2001). Its anti-inflammatory properties have been ascribed to the inhibition of the release of the pro-inflammatory cytokine TNF-α through the inhibition of the activation of the nuclear transcription factor NFκB (Wahl *et al.*, 1998), which controls, among various genes, the

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transcription of TNF-α. Beyond its anti-inflammatory action, SSZ has been assigned an anti-angiogenic potential, however, a mechanistic basis for this effect was not revealed.

In this study we show that exposure of the human monocytic/macrophage cell lines THP1 and U937 to SSZ results in a complete down-regulation of PD-ECGF/TP and IL-8. Furthermore, these cells did no longer respond to stimuli (TNF-α or IFN-γ) which normally induce PD-ECGF/TP and IL-8 in parental cells. This is the first report on a drug that provokes a marked down-regulation of the pro-angiogenic factors PD-ECGF/TP and IL-8 via a mechanism that is associated with defective signaling via the TNF-α or IFN-γ receptor pathways and altered NFκB protein expression (signaling). These data also indicate that aberration in NFκB signaling may affect PD-ECGF/TP mediated activation of 5FU prodrugs.

Materials and Methods

Chemicals. RPMI 1640 and fetal calf serum (FCS) were obtained from Cambrex Bio Science (Verviers, Belgium). Sulphasalazine (SSZ) and 5'deoxyfluorouridine (5'DFUR) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hybond ECL nitro-cellulose membranes, Hyperfilm ECL and ECL (plus) detection kit were obtained from Amersham International (Buckinghamshire, UK). Polyclonal goat anti-human PD-ECGF antibodies were obtained from R&D systems (Abingdon, UK), and the secondary antibody was peroxidase-conjugated rabbit anti goat from Dako (Glostrup, Danmark). Monoclonal antibodies for NFκB/p65 and NFκB /p105/p50 were obtained from Santa Cruz (Santa Cruz, CA, USA; sc-8008 and sc-8414, respectively); for this detection a peroxidase-conjugated goat anti-mouse secondary antibody was used (Dako). Labeled antibodies against TNF receptor I (fluorescein (FITC), TNFR I) and TNF receptor II

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(phycoerythrin (PE), TNFR II) were from R&D systems and anti-IFN receptor (CD119) (PE, IFN-γR) was from BD Biosciences (San Jose, CA, USA). RNAzol was obtained from Campro Scientific (Veenendaal, The Netherlands); Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT) was from Promega (Madison, WI, USA); deoxynucleotides (dNTPs), random hexamers and Taq polymerase from Pharmacia Biotech (Roosendaal, The Netherlands). IL-8 ELISA kits, TNF-α and IFN-γ were purchased from Sanquin (CLB, Amsterdam, the Netherlands). All other chemicals were of analytical grade.

Cell lines and exposure to sulphasalazine. THP1 and U937 human monocytic / macrophage cell lines were cultured in RPMI 1640, supplemented with 10 % FCS, 2 mM glutamine, 100 µg/ml penicillin and streptomycin at 37°C in a 5 % CO_2 in a fully humidified atmosphere. Cells were seeded at an initial density of 3 x 10^5/ml and were exposed to SSZ for a period of 3 months in order to mimic the slow action of SSZ which achieves its optimal activity after a period of 6-12 weeks (Brooks 2001). Since peak plasma levels of RA patients receiving a daily dose of 2-3 gr SSZ can reach levels of 1 mM while steady state plasma levels of SSZ can reach 0.1 mM (Baggott *et al.*, 1992;Smedegard and Bjork, 1995), we exposed cells to intermediate concentrations of 0.4-0.6 mM SSZ, which caused a moderate antiproliferative effect (IC_{50}-IC_{75}) in previously unexposed cells. Cell cultures were refreshed twice weekly. Cells stably growing in 0.6 mM SSZ, designated THP1/SSZ and U937/SSZ, were used for further characterization. The SSZ-exposed cells showed no change in cell doubling time, nor did they display any apparent morphological changes. Where indicated, cells were pre-treated for 24 hr with 150 U/ml IFN-γ or 20 ng/ml TNF-α prior to seeding for the thymidine phosphorylase activity assay. Culturing continued for an additional 24 hr after which samples were harvested in order to assess IL-8 production.

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Growth inhibition assays. Antiproliferative effects of TNF-α, 5'DFUR, doxorubcin and methotrexate were analyzed by plating 1.25 x 10^5 cells in individual wells of a 24-wells plate containing 1 ml medium/well and up to 50 µl of the drug solution. Inhibition of cell growth was determined after 72 hrs drug exposure followed by viable cell counting using a hemocytometer and trypan blue exclusion. The drug concentration required to inhibit cell growth by 50 % compared to control growth is defined as IC_{50}.

***Thymidine phosphorylase activity assay*.** TP catalyzes the phosphorolysis of thymidine. TP activity was measured in intact cells by seeding 5 x 10^5 cells (4 ml / well, 6 wells plate) in the presence of a final thymidine concentration of 0.5 mM. Samples (125 µl) were taken at selected time points up to 4 hrs. Samples were deproteinized by the addition of 40 % trichloroacetic acid (TCA), neutralized and analyzed for thymine formation by HPLC as described previously (van Triest *et al.*, 2000;Laurensse *et al.*, 1988). For each time point in the enzyme activity curves, a correction was made for volume and cell loss due to previous sampling. The reaction was linear over the entire incubation period (4 hr). TP activity is given as nmol thymine produced / 10^6 cells /hr.

mRNA analysis. RNA was extracted from 5 x 10^6 cells by the RNAzol™ method, examined for DNA contamination and reverse transcribed using random hexamers as described by the manufacturer with minimal modifications(De Bruin *et al.*, 2003).

Oligonucleotide primers used for RT-PCR of TP, IL-8 and β-actin were those previously described (Jauneau *et al.*, 2003;De Bruin *et al.*, 2003). Briefly, cDNA samples were amplified in a MJ Research PTC-2000 apparatus (Biozym, Landgraaf, the Netherlands) with 1 minute steps of denaturation at 94 ºC, primer annealing at 58 º C and elongation at 72 ºC for 35 cycles starting with a hot start at 94 ºC. PCR products were separated by 120 V electrophoresis for 2 hrs on 2 % agarose gel containing 0.1 µg / ml ethidium

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bromide. The assay has been optimized such that both target and reference genes are in the exponential phase of amplification (Rots *et al.*, 2000;van der Wilt *et al.*, 2003;De Bruin *et al.*, 2003).

Western blot analysis. For determination of TP protein expression, logarithmic growing cells were harvested and cell pellets were lysed in a lysis buffer (1 % Triton X-100; 150 mM Tris HCL, pH 7,6; 5 mM EDTA), sonicated and centrifuged for 10 min 14,000 x g at 4ºC. Protein content was assayed using the BioRad assay (BioRad Laboratories, Richmond, CA). Thirty µg protein of each sample was separated on a 12.5 % SDS-PAGE and electroblotted onto a Hybond ECL nitrocellulose membrane. Membranes were incubated overnight at room temperature in a blocking buffer containing: 1 % bovine serum albumin, 1 % milk powder in TBS-T (10 mM Tris-HCL pH 8.0, 0.15 M NaCl; 0.05 % Tween-20). The membranes were then incubated with the primary antibody (goat anti-human PD-ECGF at a 1:1000 dilution), followed by incubation with horseradish peroxidase conjugated rabbit anti-goat antibody (1:2000). Nuclear and cytoplasmic fractions were prepared using the NE-PER nuclear and cytoplasmic extraction reagents from Pierce Biotechnology (Rockford, IL, USA), according to manufacturer's protocol. Thirty µg of nuclear and cytoplasmic fractions were separated on a 10 % SDS-PAGE, following electoblotting, the membrane was probed with NFκB/p65 or NFκB/p105/p50 antibodies (mouse anti-human at a 1:500 dilution) followed by peroxidase-conjugated goat anti-mouse antibody (1:3000). Proteins were visualized using the enhanced chemiluminescence detection kit (ECL plus) and Hyperfilm.

IL-8 ELISA. Cellular release of IL-8 was analyzed by ELISA, and was performed according to manufacturer's protocol with minimal modifications (lower limit of detection;

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5 pg / ml). Samples were taken from the same wells in which the TP activity assay was performed. For comparison a similar set of wells was tested without adding TdR.

Expression of TNFR I, TNFRII and IFN-γR. THP1, THP1/SSZ, U937 and U937/SSZ cells were labeled with FITC/PE-conjugated antibodies against TNFR I, TNFR II and IFN-γR or their appropriate isotype controls. Subsequently, the cells were analyzed by flow cytometry using a FACS-calibur flow cytometer (Becton and Dickinson, Erembodegem-Aalst, Belgium). Data analysis was performed using Cellquest software package.

Statistics. The one-tailed, paired Student's t-test was used to determine the difference in TP activity and IL-8 production, in untreated controls versus IFN-γ or TNF-α treated cells.

Results

Characterization of SSZ-exposed cells. Relative to parental cells, the THP1/SSZ and U937/SSZ variants displayed a 2- and 3.3 fold increase in the IC_{50} for SSZ, respectively. Following the phenotypic characterization of SSZ-exposed THP1 and U937 cells, we observed a marked resistance to 5'DFUR and TNF-α in these SSZ-treated variants (Table 1). For THP1/SSZ and U937/SSZ cells, no IC_{50} was reached at the highest concentration of 5'DFUR and TNF-α tested; these cell lines displayed relative 5'DFUR-resistance levels of > 138.8- and > 89-fold, respectively. Furthermore, these cell lines were >34.5- and >40-fold resistant to TNF-α, respectively. This resistant phenotype was retained when SSZ-exposed cells were grown in the absence of SSZ for at least one month, suggesting that the observed effects were relatively stable (data not shown). In order to address whether these phenotypic changes relate to SSZ-induced

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targeting of NFκB, we also determined the growth inhibitory effects of 2 chemotheapeutic drugs, the folate antagonist methotrexate (MTX) and doxorubicin, mechanistically unrelated to TNFα and 5'DFUR. No significant differences for parental THP1 versus THP1/SSZ and parental U937 cells versus U937/SSZ were observed in growth inhibitory potential of MTX (IC_{50}: 9.3 ± 1.6 nM vs 10.1 ± 2.9 nM, and 7.3 ± 2.9 nM vs 8.3 ± 0.7 nM, respectively, mean ± SD of 3 experiments) and doxorubicin (IC_{50}: 20.4 nM vs 21.6 nM, and 12.9 nM vs 10.6 nM, respectively, mean of 2 experiments).

PD-ECGF/TP and IL-8 mRNA and PD-ECGF/TP Western blot analysis. A plausible explanation for the 5'DFUR resistance in SSZ-exposed cells could be down regulation of PD-ECGF/TP expression/activity since TP activity is required for the conversion of 5'DFUR to 5FU (De Bruin *et al.*, 2003). In this respect, TNFα resistance may also have implications for alterations in PD-ECGF/TP expression/activity since TNFα is a known positive regulator/inducer of PD-ECGF/TP (Eda *et al.*, 1993). To investigate this and the possible co-regulation of IL-8, another macrophage produced angiogenic factor, PD-ECGF/TP and IL-8 gene expression was determined. In THP1/SSZ and U937/SSZ cells PD-ECGF/TP mRNA levels were below the limit of detection (Figure 1A). Likewise, a marked down-regulation of IL-8 mRNA was observed in THP1/SSZ and U937/SSZ cells (Figure 1 A). Consequently, Western blot analysis revealed that PD-ECGF/TP protein was below the level of detection (Figure 1 B).

Regulation of PD-ECGF/TP activity and IL-8 production. To determine a potential mechanism of down-regulation and co-regulation of PD-ECGF/TP and IL-8, wild type THP1 and U937 cells as well as SSZ-exposed cells were incubated with cytokines known to induce and possibly regulate PD-ECGF/TP (IFN-γ and TNF-α) or IL-8 (TNF-α) (Figure 2 A, B). In THP1/SSZ and U937/SSZ cells exposed to SSZ for 3

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months, TP-activity was not measurable. Remarkably, when THP1 cells were exposed to 0.6 mM SSZ for 1 week and 2 weeks, a modest decrease in TP activity (29% and 37%, respectively) could be already observed, suggesting that the decline in TP activity is rather gradual, which may be correlated with the slow action of SSZ.

IFN-γ and TNF-α significantly up-regulated TP activity in THP1 cells 2.1-fold and 1.5-fold, respectively. Similarly, TP activity was significantly increased in U937 cells after stimulation with IFN-γ (2.8-fold) and TNF-α (1.7-fold). In both parental cell lines there was a significantly increased TP activity after IFN-γ treatment when compared with TNF-α treated cells. Effects of TNF-α or IFN-γ on IL-8 release were consistent with the effects on TP; a 1.8- and 1.6-fold increase in IL-8 release from THP1 cells treated with IFN-γ or TNF-α, respectively. IL-8 production in U937 cells increased 1.6- fold and 1.2 fold after treatment with IFN-γ and TNF-α, respectively. There was no significant difference in IL-8 production in parental cells used for the TP activity assay, which were incubated with TdR for 24 hr, compared to cells which were incubated for 24 hr in the absence of TdR (data not shown). Strikingly, no detectable TP activity or IL-8 release was observed in THP1/SSZ and U937/SSZ cells. Moreover, both TNF-α and IFN-γ failed to increase TP activity and IL-8 release from SSZ-exposed cells.

TNFRI, TNFRII and IFN-γR expression and NFκB /p65, NFκB /p105/p50 Western blot analysis. SSZ-exposed cells were resistant to TNF-α, and IFN-γ or TNF-α could no longer induce PD-ECGF/TP and IL-8 in THP1/SSZ and U937/SSZ cells. To examine whether this apparently aberrant signaling is possibly due to alterations in the levels of cytokine receptors, TNFRI, TNFRII and IFN-γR expression was assessed using flow cytometry. All three receptors were expressed on the surface of THP1 and U937 cells, although there was a heterogeneous expression of TNFRII on THP1 cells. No

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major change in IFN-γR expression was observed; however, a decrease in TNFRI levels and a complete down regulation of TNFRII was noted in SSZ-exposed cells (Figure 3 A).

Since SSZ targets the NFκB signaling pathway, possible changes were analyzed by studying three NFκB family members, p65 and p105/p50, at the level of protein expression. In the SSZ-exposed cells there was a down regulation of p105 in the cytoplasm, accompanied by a down regulation of p50 in the nuclear fraction when compared to parental cells. There were no apparent differences in the cytoplasmic p65. Surprisingly however, whereas parental cells contain no detectable levels of p65 in the nucleus, SSZ-exposed cells displayed a constitutive presence of substantial p65 levels in the nucleus (Figure 3 B)

Discussion

In this paper we describe the marked down-regulation of two macrophage produced pro-angiogenic factors, PD-ECGF/TP and IL-8, in THP1 and U937 monocytic cell lines following exposure to SSZ. In addition, SSZ-exposed cells displayed resistance to TNF-α. Importantly, consistent with the down-regulation of PD-ECGF/TP was the high level of resistance to 5'DFUR which requires a prior activation to 5FU by PD-ECGF/TP in order to elicit its cytotoxic activity. Thus, prior pretreatment with SSZ may provoke resistance to capecitabine, a prodrug for 5'DFUR and ultimately 5FU.

The exact mechanism of regulation of PD-ECGF/TP gene expression is yet unknown, however the promoter contains several Sp1 transcription factor binding sites (i.e. GC-box) postulated to contribute both to the basal and TNF-α-inducible expression (Zhu *et al.*, 2002). Furthermore, interferon-mediated signaling via the signal transducer and activator of transcription (STAT) family of transcription factors occurs at two

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potential binding sites; the IFN-stimulated response element (Schwartz *et al.*, 1998) and the gamma activated sequence (Goto *et al.*, 2001). Since PD-ECGF/TP and IL-8 transcripts and proteins were absent or markedly reduced in SSZ-exposed cells, we investigated known pathways, which can induce the expression of these factors. IFN-γ is a potent inducer of PD-ECGF/TP expression, whereas TNF-α induces both PD-ECGF/TP (Eda *et al.*, 1993) and IL-8 gene expression (Hoffmann *et al.*, 2002). Consistent with previous studies (Eda *et al.*, 1993) (Goto *et al.*, 2001), we observed that IFN-γ and TNF-α were able to induce PD-ECGF/TP expression in both parental THP1 and U937 cells. IL-8 was also inducible by both cytokines in parental cell lines. IFN-γ and TNF-α appear to be common stimuli for PD-ECGF/TP and IL-8 expression in parental cells. Although IFN-γ is not commonly associated with IL-8 induction, it has been previously described that IL-8 induction can occur through a post-transcriptional mechanism (Bosco *et al.*, 1994), while PD-ECGF/TP induction by IFN-γ is thought to be mediated directly via STAT1 signaling (Goto *et al.*, 2001). However, in the SSZ-exposed cell lines, PD-ECGF/TP and IL-8 were neither expressed nor induced by these cytokines.

Taken together, these results suggest aberrant signaling and regulatory pathway(s) in the SSZ-exposed cells, which was further reinforced by the down-regulation of TNFRI and TNFRII expression in SSZ-exposed cells. The expression of IFN-γR was largely unchanged for both SSZ-exposed cell lines, indicating that the putative defect would presumably lie downstream of the receptor. Although TNFRI is associated with cell death, it has been shown that TNFRII can exert an effect on cell death via TNFRI (Gupta, 2002). Furthermore, it has been described that TNFRII is more frequently a subject to regulation (Gupta, 2002). As such, the down-regulation of TNFRI

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and the loss of TNFRII could explain the resistance to TNF-α induced cell death in the SSZ-exposed cells (Table 1). The down-regulation of both TNF receptors could also function as part of an efficient anti-inflammatory response mechanism exerted by SSZ, inhibiting the action of TNF-α. The present study suggests that TNF receptors and their ligands may play a role in the basal level of PD-ECGF/TP and IL-8 expression and exposure to SSZ may disrupt these signaling pathways. The down-regulation of angiogenic factors is another property of SSZ in anti-rheumatic and related inflammatory diseases. Of note, treatment of (chronic) inflammatory bowel disease with SSZ, conferred at least some protection against the development of colon cancer in these patients (Ryan *et al.*, 2003). The decribed down-regulation of angiogenic factors might play a role in this chemoprotective trait of SSZ.

SSZ has been shown to act as an NFκB inhibitor, by blocking the phosphorylation of IκBa (Wahl *et al.*, 1998). The present study reveals that prolonged inhibition of the NFκB pathway signaling by SSZ ultimately resulted in an anti-angiogenic response, illustrated by PD-ECGF/TP and IL-8 down-regulation. Our present data suggest that impaired NFκB transcriptional activation may be the underlying mechanism for this effect. In particular, an enhanced nuclear accumulation of NFκB/p65 was noted in the SSZ exposed cells compared to the parental cells, however, one of its heterodimerization partners necessary for transcriptional activity, NFκB/p50, was not up-regulated (Perkins, 2000). This stoichiometric imbalance can possibly impair the formation of transcriptionally-active p65/p50 heterodimers. This will necessarily result in a transcriptional dysfunctioning of the NFκB pathway. NFκB directly regulates IL-8, and the TNFRs utilize NFκB pathway in their signal transduction. No direct involvement of NFκB has been shown in relation to PD-ECGF/TP expression although in a recent

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paper, Zhu et al (Zhu and Schwartz, 2003) suggested that NFκB and TNFRII may be involved in the regulation of PD-ECGF/TP gene expression .

Although this study is focussed primarily on the role of PD-ECGF/TP in angiogenesis, the outcome of this study may have major implications for the chemotherapeutic efficacy of the 5FU prodrug capecitabine (Miwa *et al.*, 1998). Currently capecitabine is the most widely prescribed oral drug for the treatment of colorectal cancer, but requires activation by PD-ECGF/TP. Since exposure to SSZ down-regulates TP activity this leads to resistance to capecitabine (Table 1). In numerous studies PD-ECGF/TP has been shown to co-localize with tumor associated macrophages (Takahashi *et al.*, 1996;Toi *et al.*, 1999). Consequently, PD-ECGF/TP from macrophages can be responsible for increased 5FU accumulation and enhanced efficacy, due to a bystander effect (Evrard *et al.*, 1999). However, when prior treatment with NFκB interacting drugs down-regulates PD-ECGF/TP, this may negatively affect the efficacy of capecitabine. Therefore these patients should be carefully screened for their TP level in macrophages and tumor cells before receiving capecitabine. In addition, future studies should also evaluate TP expression in macrophages of patients with RA treated with SSZ for a prolonged period.

To our knowledge this is the first example of a treatment (i.e. SSZ) that results not only in the down-regulation of PD-ECGF/TP but also in a complete inhibition of pathways known to up-regulate PD-ECGF/TP. The results were observed in two independent monocytic / macrophage cell lines. We propose that the down-regulation of PD-ECGF/TP and IL-8 may be the consequence of a non-functional (auto)crine loop due to changes in TNF-receptor expression and downstream signaling pathways of both IFN-γ and TNF-α receptors, in combination with an altered NFκB pathway. Hence, NFκB and TNF(R)-mediated signaling pathways warrant further attention as targets for future

optimization of anti-angiogenic therapy, focussing on their role in the functioning of macrophages and the expression of PD-ECGF/TP and IL-8.

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Footnotes

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Figure 1. **A**) Representative agarose gel of separated RT-PCR products of TP, IL-8 and β-actin, showing the complete loss of PD-ECGF/TP and the marked decrease of IL-8 m-RNA in the SSZ-exposed cells. Even higher cDNA concentrations in the PCR reaction did not lead to any detectable bands for PD-ECGF/TP, in line with a complete disappearance of PD-ECGF/TP expression. **B**) Western blot of PD-ECGF/TP showing the markedly diminished PD-ECGF/TP protein expression in the SSZ-exposed cells. β-actin was included as a loading control.

Figure 2. TP activity and IL-8 production in parental and SSZ-exposed cells after incubation with various cytokines. Cells were treated for 24 hr with IFN-γ (150 U / ml) or TNF-α (20 ng / ml), then seeded at a density of 0.5 x 10^6 cells / 4ml, samples were taken over a 4 hr period to determine the TP-activity, followed by another period of 24 hr at which point the samples were taken for IL-8 determination. **A**) TP activity for the control and treated, parental and SSZ-exposed cells. Note, no TP activity is detected in the exposed cells. **B**) the same cells used to measure the TP activity were cultured for an additional 24 hr to assess the IL-8 production. There was no measurable IL-8 production in the SSZ-exposed cells. * $P < 0.05$ for control vs IFN-γ or TNF-α treatment. Results are the means of 3 separate experiments ± S.E.M, # $P < 0.05$ for IFN-γ vs TNF-α treatment, nd: not detectable.

Figure 3. **A**) Representative picture of a flow cytometry experiment assessing the expression of IFN-γR TNFRI, and TNFRII in parental and SSZ-exposed THP1 and U937 cells. Results show that IFN-γR was expressed at similar levels, TNFR I was down-regulated and there was a complete loss of TNFRII. The mean fluorescence (specific

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signal minus isotype control signal) for TNFRII decreased from 36 to 2 and 60 to 3 for THP1 and U937 compared to their SSZ-exposed counterparts, respectively. Dotted line: isotype control (autofluorescence) black fill: specific signal.

B) Western blot of NFκB family members: p105/50 and p65 in cytoplasmic and nuclear fractions of parental and SSZ-exposed THP1 and U937 cells. Results show a minor decrease in cytoplasmic p105 and a decrease of nuclear p50 in the SSZ-exposed cells compared to parental cells. No apparent differences in cytoplasmic p65 levels were observed. In contrast, constitutive p65 expression was found in the nuclear fractions of SSZ-exposed cells, which was absent in the parental cells. The p105/50, p65 and β-actin, are all from one representative experiment and blotted on three separate gels; from each gel the bands were rearranged to better compare the nuclear and cytoplasmic content.

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Table 1. Cell growth inhibitory effects **(**IC_{50}) values of the fluoropyrimidine 5'DFUR and TNF-α in the THP1 and U937 monocytic cell lines and their sulphasalazine-exposed variants, THP1/SSZ and U937/SSZ.

Cell line	5'DFUR (μM)	RR	TNF-α (ng/ml)	RR
THP1	1.8 ± 0.5		2.9 ± 0.1	
THP1/SSZ	> 250	>138.8	> 100	>34.5
U937	2.8 ± 0.2		2.3 ± 0.7	
U937/SSZ	> 250	>89	> 100	>40

RR is the relative resistance (SSZ-exposed variant IC_{50}) / (parental IC_{50})

Results are the mean ± S.E.M. of 3 separate experiments

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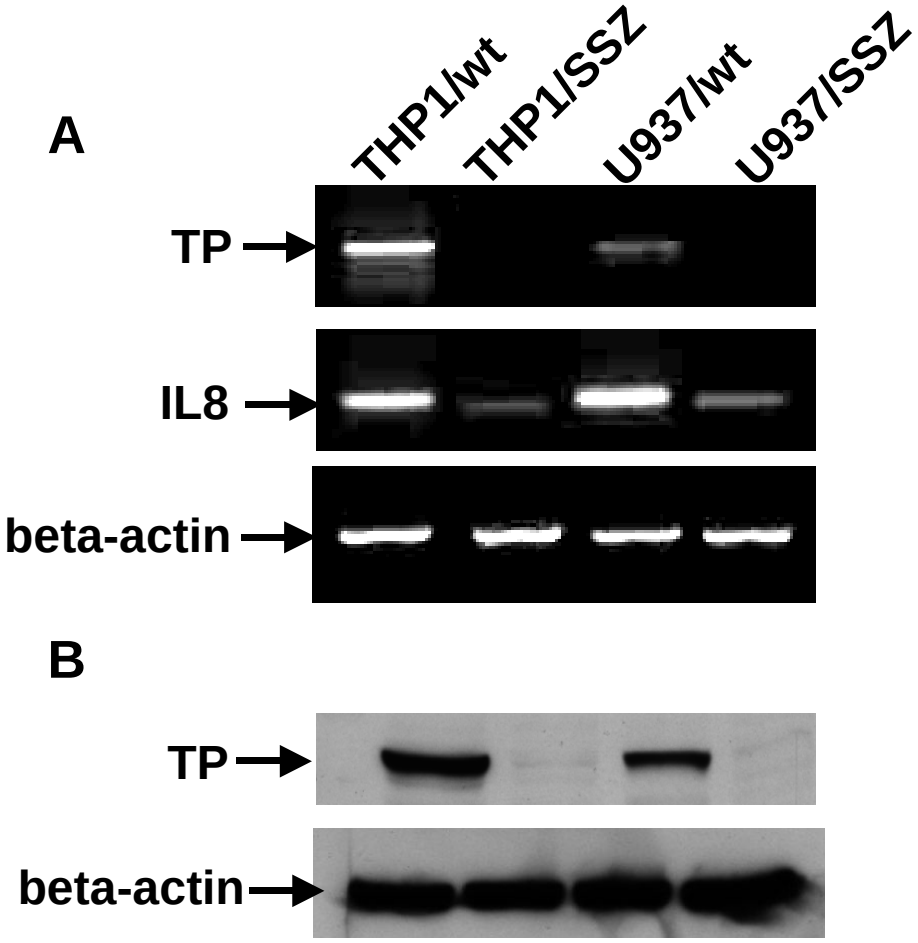

Figure 1

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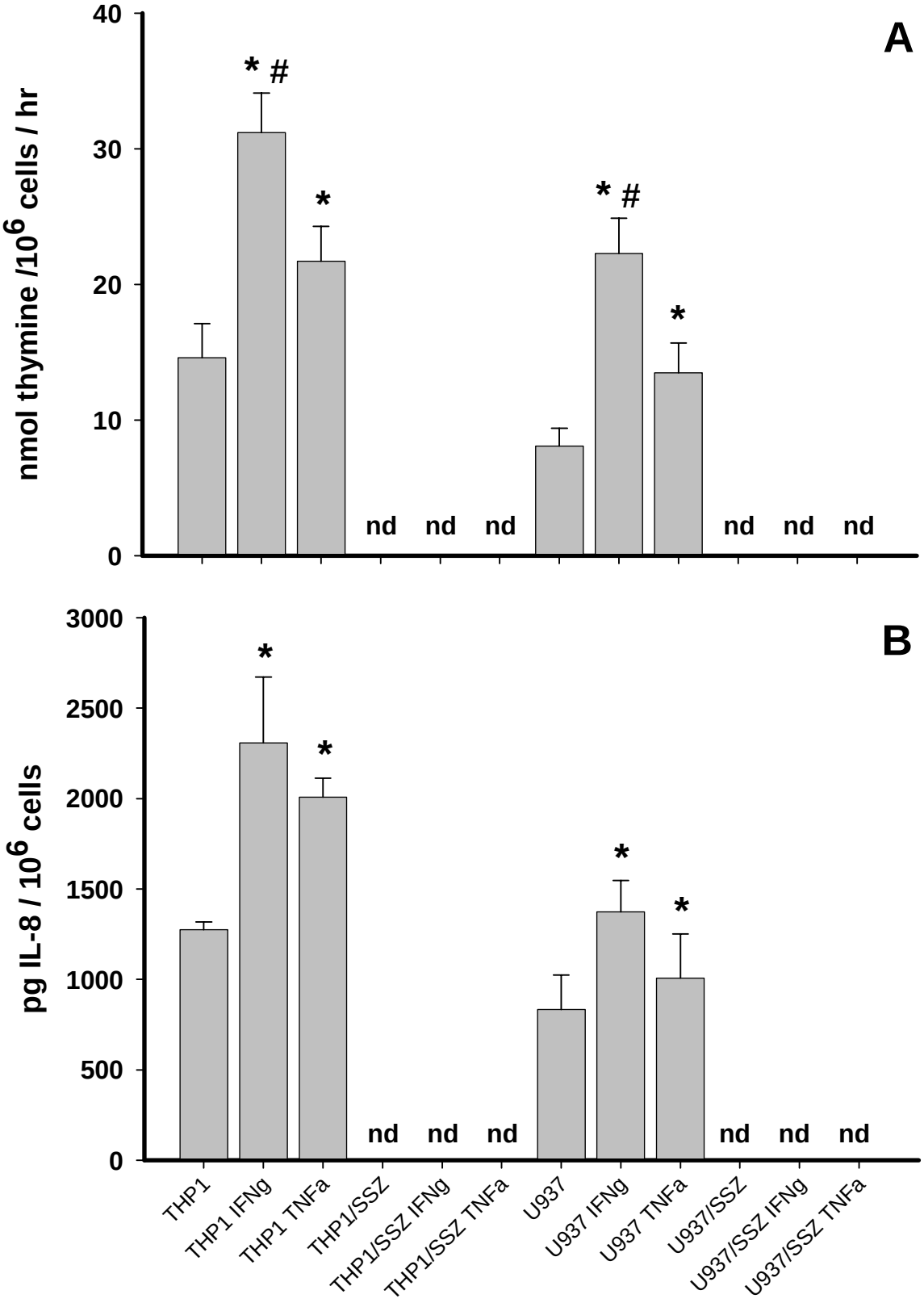

Figure 2

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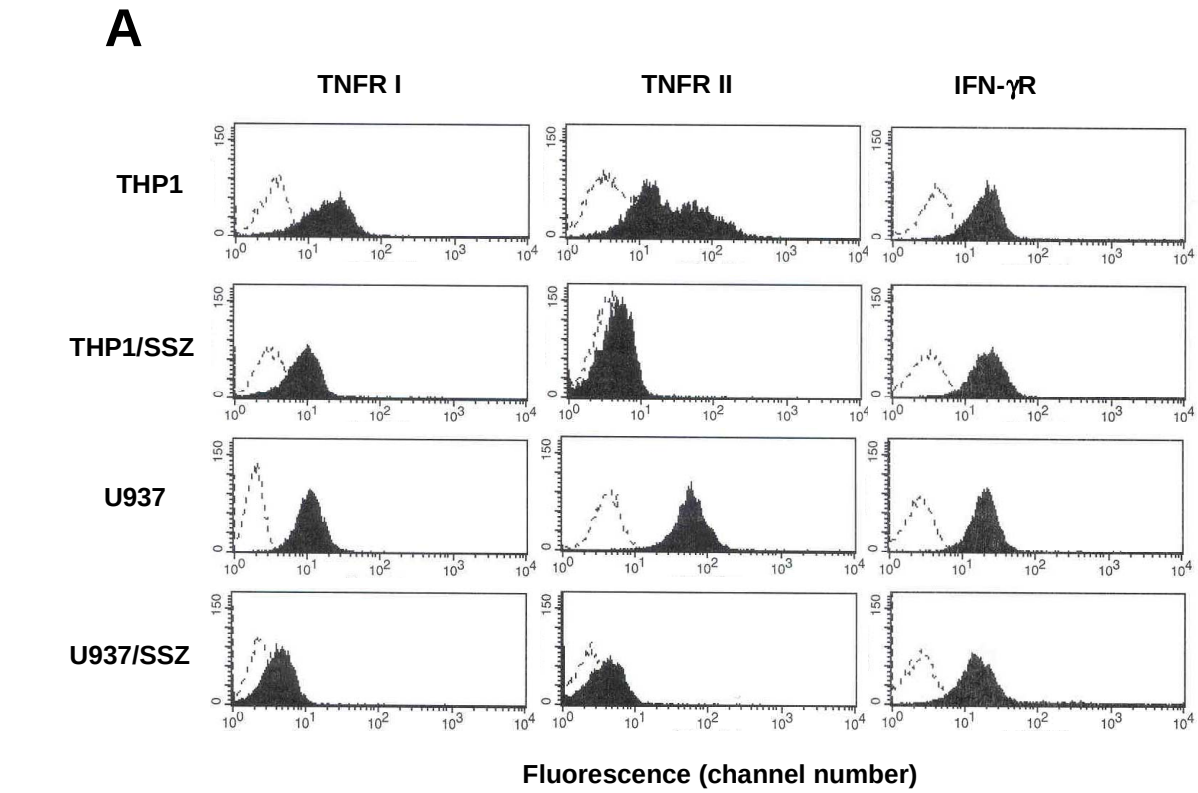

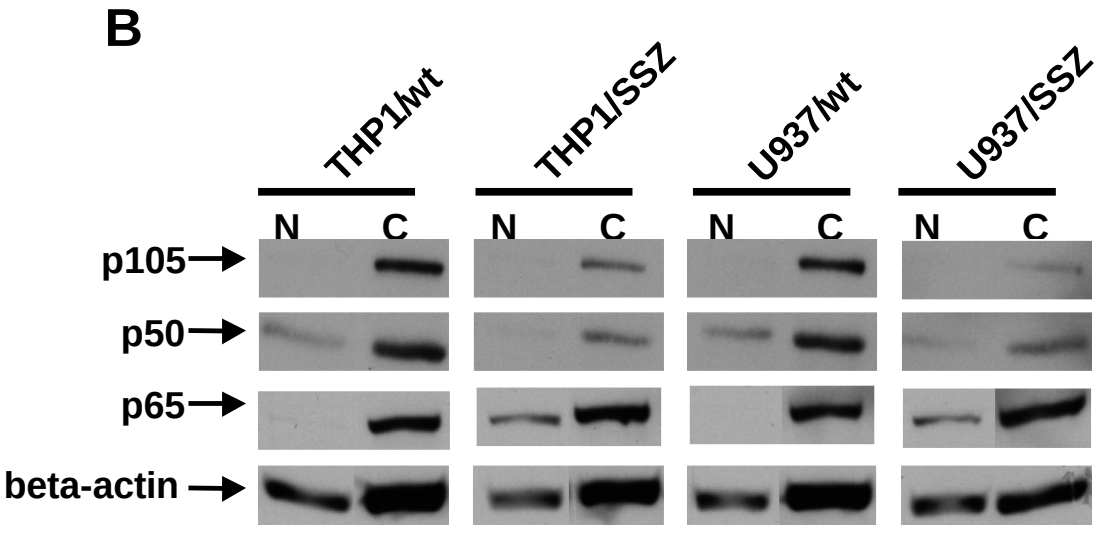

Figure 3