

# Activation of respiratory complex II by interferon-gamma and its inhibition by pyrimidine derivatives

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## Abstract

**OBJECTIVES:** Formation of formazan is a commonly used measure of cytotoxicity of compounds. It is a product of reduction of tetrazolium salts such as 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride. The extent of substrates reduction reflects the activity of enzymes succinate dehydrogenase (SDH; respiratory complex II) and lactate dehydrogenase (LDH), respectively. The aim of present study was a) to investigate formazan formation under the conditions of in vitro stimulation of cells with interferon- $\gamma$  (IFN- $\gamma$ ) and lipopolysaccharide (LPS), and b) to analyse possible interference of pyrimidine analogues with formazan production.

**METHODS:** Peritoneal cells and splenocytes were obtained from C57BL/6 mice. They were cultured at 37 °C, 5% CO<sub>2</sub> in humidified incubator. Levels of formazan were determined at the interval of 24 h of culture using the WST-1 and LDH assays. Nitric oxide (NO) was activated by IFN- $\gamma$  plus LPS and assayed by Griess reagent 24 h afterwards. Pyrimidines were applied concomitantly with immunostimulatory agents.

**RESULTS:** IFN- $\gamma$  enhanced concentration of SDH-produced formazan by macrophages (not by splenocytes) by approximately 50%. The activity of LDH remained unaffected. LPS was ineffective in both cases. While pyrimidines with NO-inhibitory properties suppressed the IFN- $\gamma$ -enhanced levels of SDH-produced formazan, they did not change the LDH-dependent formazan production.

**CONCLUSION:** IFN- $\gamma$  augments the SDH-produced formazan by macrophages. It does not change the LDH-dependent formazan formation. The enhancing effect may have a significant impact upon the appropriate interpretation of cytotoxic properties of drugs investigated under the conditions of immune stimulation of cells.

## Abbreviations:

IFN-	- interferon-	SDH	- succinate dehydrogenase (E.C. 1.3.99.1); respiratory complex II
IL-	- interleukin-	STAT	- signal transducer and activator of transcription
JAK	- Janus kinase	TNF- $\alpha$	- tumour necrosis factor- $\alpha$
LDH	- lactate dehydrogenase (E.C. 1.1.1.27)	WST-1	- tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
LPS	- lipopolysaccharide		
NO	- nitric oxide		

## INTRODUCTION

Ubiquitously occurring mitochondrial complex II, also known as succinate:quinone oxidoreductase or succinate dehydrogenase (SDH), is one of mitochondrial respiratory enzymes of the Krebs cycle implicated in the electron transport chain. SDH oxidizes succinate to fumarate and reduces ubiquinone to ubiquinol in the respiratory chain. The ability of SDH to reduce artificial substrates such as tetrazolium salts to coloured formazan products (Slater *et al.* 1963) has become a basis for the development of quantitative colorimetric assay which is frequently used in order to assess the cytotoxicity of compounds. It has been shown that only viable cells with metabolically active mitochondria convert tetrazolium salts to formazan (Mosmann 1983). The most commonly used tetrazolium salt is a water soluble 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1).

Another enzyme-rooted cytotoxicity assay reflects damaged plasma membrane integrity resulting in the release of enzymes including lactate dehydrogenase (LDH) from cells in culture medium. LDH enzyme catalyzes the reversible conversion of pyruvate to lactate using NAD<sup>+</sup> as a cofactor. The production of eventual NADPH can be determined by the reduction of tetrazolium salts, *e.g.* 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride into formazan (Decker & Lohmann-Matthes 1988).

Evaluation of cytotoxicity of compounds is an indispensable requirement in pilot studies screening for their biological, pharmacologically prospective properties. It is recognized that expression of cytotoxic effects may be influenced by a number of experimental variables (Groh *et al.* 2012; Horváthová *et al.* 2006). Conceivably, any interference with the activity of enzymes may bring about a bias in estimating the viability of cells. The aim of present experiments was to investigate the production of formazan under the conditions of immune stimulation of cells with interferon- $\gamma$  (IFN- $\gamma$ ) and/or bacterial lipopolysaccharide (LPS). Effects of these immunostimulatory agents were evaluated using the WST-1 and LDH assays. Possible impact of the immune activation of SDH on interpretation of cytotoxic properties of compounds was demonstrated using recently described derivatives of pyrimidine with distinct NO-inhibitory properties (Jansa *et al.* 2014).

## MATERIAL AND METHODS

### Origin of cells and cell culture microenvironment

Female mice of the inbred strain C57BL/6, 8–10 wks old, were purchased from Charles River Deutschland (Sulzfeld, Germany). They were kept in transparent plastic cages in groups of ten. Lighting in animal room was set on 06 to 18 h, temperature at 22 °C.

Pooled peritoneal cells collected from mice (n=5–8 in individual experiments) were washed, resuspended

in culture medium, and seeded into 96-well flat-bottom microplates (Costar). Adherent cells (macrophages) were isolated by incubating the cells for 2 h at 37 °C, 5% CO<sub>2</sub>, and then vigorously shaking the plate and washing the wells three times to remove non-adherent cells.

Single-cell suspension of splenocytes was prepared by passing fragmented pooled spleens through a fine nylon sieve. Erythrocytes were removed using the red blood cells lysing buffer (Sigma) containing 0.83% ammonium chloride in 0.01 M Tris-HCl, pH 7.5. After repeated thorough washing the cells were seeded in quadruplicate wells of 96-well flat-bottom culture plates (Costar).

The WST-1 and LDH toxicity assays were run using  $0.5 \times 10^5$  cells/well/100  $\mu$ l, if not stated otherwise. Production of NO was determined in cultures of  $2 \times 10^5$  well/100  $\mu$ l.

RPMI-1640 culture medium (Sigma-Aldrich) containing 2 mM L-glutamine, 50  $\mu$ g/ml gentamicin, and  $5 \times 10^{-5}$  M 2-mercaptoethanol (all Sigma) was used. In order to avoid high background OD values in the LDH assay, the foetal bovine serum was excluded. Its absence had no effect on the performance of NO and cytotoxicity assays (data not shown). Cultures were maintained at 37 °C, 5% CO<sub>2</sub> in 100% humidified Heraeus incubator.

The animal welfare and all experimental procedures have been approved by the Institution Animal Ethics Committee.

### Formazan production

WST-1 and LDH kits were purchased from Roche Diagnostics (Mannheim, Germany) and Sigma-Aldrich (Prague, CZ), respectively. If not stated otherwise (as specified in Results), the content of formazan in supernatants of cells was determined at the interval of 24 h of culture. Triton (1%) was used to induce 100% cell death in reference settings. After addition of WST-1, the cells were kept in the Heraeus incubator at 37 °C for additional 3 h. Optical density at 450/690 nm was evaluated. LDH-dependent formation of formazan was determined after 30-min incubation in the dark at ambient temperature. The reaction was stopped with 2 N HCl. Differences between absorbances at 492 and 690 nm were evaluated. The absorbances were recorded using a microplate spectrophotometer (Tecan, Austria).

### NO assay

Cells were cultured 24 h in the presence of NO-priming immune stimuli, *i.e.* murine recombinant IFN- $\gamma$  (R&D Systems, Minneapolis, MN) and LPS (*Escherichia coli* 0111:B4; Sigma). The concentration of nitrites in supernatants of cells was detected in individual, cell-free samples (50  $\mu$ l) incubated 5 min at ambient temperature with an aliquot of a Griess reagent (1% sulphanilamide/0.1% naphthylendiamine/2.5% H<sub>3</sub>PO<sub>4</sub>). The absorbance at 540 nm was recorded using a microplate spectrophotometer (Tecan). A nitrite calibration curve was used to convert absorbance to  $\mu$ M nitrite.

### Pyrimidine derivatives

Based on the recently published data on the interference of several pyrimidines with the inducible high-output NO production (Jansa *et al.* 2014), the following groups of derivatives have been selected for the present study: 2-amino-4,6-dihydropyrimidines (group I), 2-amino-4,6-dichloropyrimidines (group II), and 4,6-dichloro-2-[(*N,N*-dimethylamino)methyleneamino]pyrimidines (group III). Each group is represented by compounds substituted at position C-5 of the pyrimidine ring with phenyl (1) or benzyl (2) group. All tested compounds were synthesized in-house by P.J. (IOCB).

### Statistical analysis

Analysis of variance (ANOVA), subsequent Bonferroni's multiple comparison test, and graphical presentation of data were done using the Prism program (GraphPad Software, San Diego, CA).

## RESULTS

### Effects of IFN- $\gamma$ on WST-1- and LDH-assayed accumulation of formazan in cell supernatants

Supernatant concentrations of formazan increased steadily in dependence on increasing number of cells in plate well (Figure 1A). The increase was statistically highly significant irrespective of the WST-1 ( $F_{3,24}=80.81$ ,  $p<0.0001$ ) and LDH ( $F_{3,24}=98.24$ ,  $p<0.0001$ ) detection system used.

The amount of the SDH-produced formazan was substantially enhanced upon the exposure of cells to IFN- $\gamma$  ( $F_{1,24}=107.7$ ,  $p<0.0001$ ). The increment ranged from 34.6% ( $p<0.05$ ) to 48.4% ( $p<0.0001$ ) for the cell amount of  $25 \times 10^3$ /well and  $50 \times 10^3$ /well, respectively (Figure 1B).

In contrast, IFN- $\gamma$  did not influence ( $F_{1,24}=0.03$ ,  $p=0.86$ ) formazan levels determined by means of LDH assay (Figures 1A, 1B).

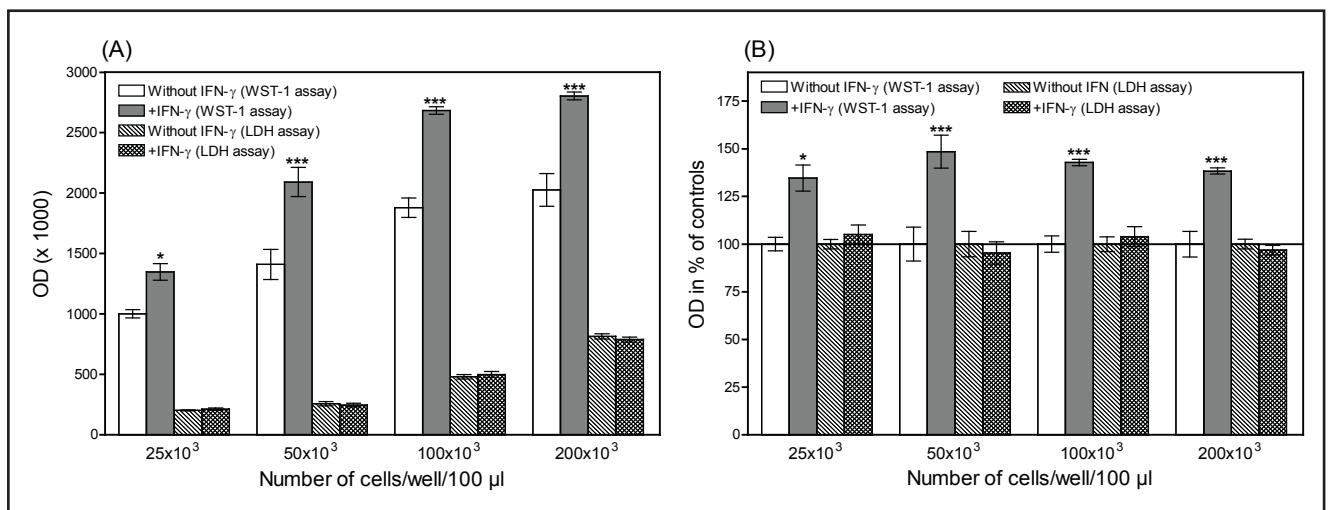
Several independent experiments proved reproducibility of the effects (Figure 2A). The absolute OD levels of control SDH-produced formazan differed among individual experiments ( $F_{1,73}=21.01$ ,  $p<0.0001$ ). The over-all average OD value was  $1.459 \pm 0.163$  ( $\pm 95\%$  limits of confidence). With no exception, the formazan levels were enhanced by IFN- $\gamma$  and were closely correlated with those found in control (*i.e.* IFN- $\gamma$ -untreated) cells ( $r_{12}=0.88$ ,  $p<0.001$ ). Transformed to the percentage with respect to the controls (Figure 2B), formazan was on average enhanced by  $39.9 \pm 7.8\%$  (mean  $\pm 95\%$  limits of confidence). The difference between control *versus* IFN- $\gamma$ -treated cells was statistically highly significant ( $F_{1,121}=438.1$ ,  $p<0.0001$ ).

Also concentration of LDH-assayed formation of formazan differed among experiments (Figure 2A), the average OD value being  $0.240 \pm 0.040$ . In this case, it remained uninfluenced ( $F_{1,102}=0.0003$ ,  $p=0.987$ ) by IFN- $\gamma$  (Figures 2A, 2B).

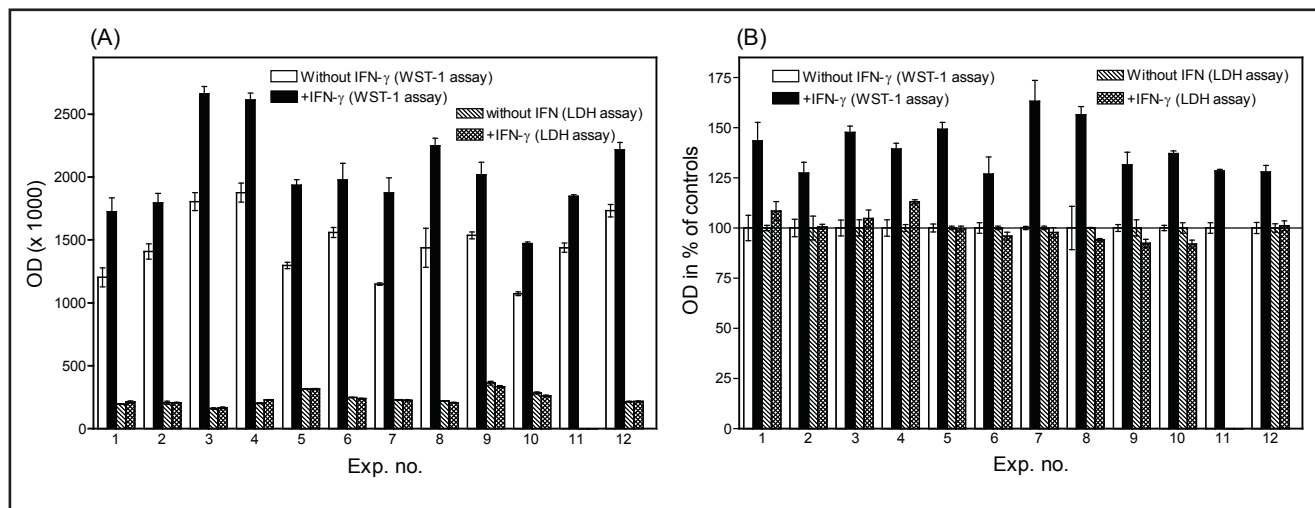
### Dependence of IFN- $\gamma$ -enhancing effects on the type of cells

Levels of SDH-produced formazan in supernatants of the whole population of peritoneal cells did not differ ( $F_{1,56}=7.50$ ,  $p=0.008$ ) from those produced by adhered subpopulation of cells (Figure 3). The adhered cells were prepared from the same cell suspension and grown under identical experimental conditions. No significant difference ( $p>0.05$ ) was observed between the enhancing influence of IFN- $\gamma$  in both variants of peritoneal cell cultures.

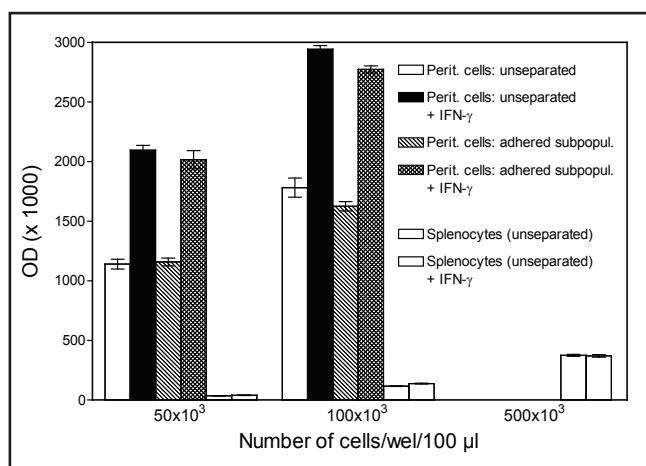
Splenocytes showed very low absorbance signal (Figure 3). It was negligible with the suspension of



**Fig. 1.** Supernatant accumulation of formazan in dependence on a number of mouse peritoneal cells in microplate wells. The cells were cultured for 24 h in absence or presence of 5 ng/ml of recombinant murine IFN- $\gamma$  (A). In order to compare the effects of IFN- $\gamma$  under varying experimental conditions, the absolute OD values were expressed as a percentage of relevant control values (B). Concentration of formazan was determined by the WST-1 and LDH assays. The bars are means  $\pm$  SEM. Figures are representative of two identical experiments. Statistical significances: \*,  $p<0.05$ ; \*\*\*,  $p<0.0001$ .



**Fig. 2.** Study of reproducibility of responsiveness of mouse peritoneal cells to the influence of IFN- $\gamma$  (5 ng/ml) on accumulation of formazan in cell supernatant. The cells were grown 24 h in density of 50,000/well/100  $\mu$ l of culture plates. At least twelve experiments were run within the interval of 14 months (A). Concentration of formazan was determined using the WST-1 and LDH assays. For comparative purposes, the percent transformation of the data has been made (B). The bars are means  $\pm$  SEM. All IFN- $\gamma$ -induced elevations were statistically significant ( $p < 0.01$  to  $p < 0.0001$ ).



**Fig. 3.** Comparison of effects of IFN- $\gamma$  (5 ng/ml) on production of formazan by adhered subpopulation (macrophages) versus whole population of mouse peritoneal cells, and splenocytes. Formazan was determined after 24 h of culture using the WST-1 assay. Each bar is mean  $\pm$  SEM. The data are representative of three identical experiments.

50,000 cells/well. Only the 10-fold increased density of splenocytes (500,000/well) did show enhanced formation of formazan. Yet, it was approximately 3-times lower than the concentration reached in peritoneal cells cultured at suspension of 50,000 cells/well.

#### Dose- and time-dependent enhancing effects of IFN- $\gamma$ on SDH-produced formazan

The onset of statistically significant enhancing effect of IFN- $\gamma$  on the SDH-produced formazan was observed with as low dose as 250 pg/ml ( $p < 0.05$ ). Higher doses of IFN- $\gamma$ , up to 5000 pg/ml, were more effective ( $p < 0.01$  to  $p < 0.001$ ) (Figure 4A).

The bacterial endotoxin (LPS) was ineffective on its own to influence the formazan production ( $F_{/6,27/} = 1.30$ ,  $p = 0.30$ ). It neither increased nor decreased the augmented formazan levels triggered by IFN- $\gamma$  ( $F_{/1,30/} = 0.009$ ,  $p = 0.93$ ).

Statistically significant enhancing effect of IFN- $\gamma$  was detectable within the interval of 4 h ( $p < 0.05$ ) successive to its addition to peritoneal cells (Figure 4B). The percent increase (by 57%) was similar to that observed at the delayed interval of 27 h (by 46%).

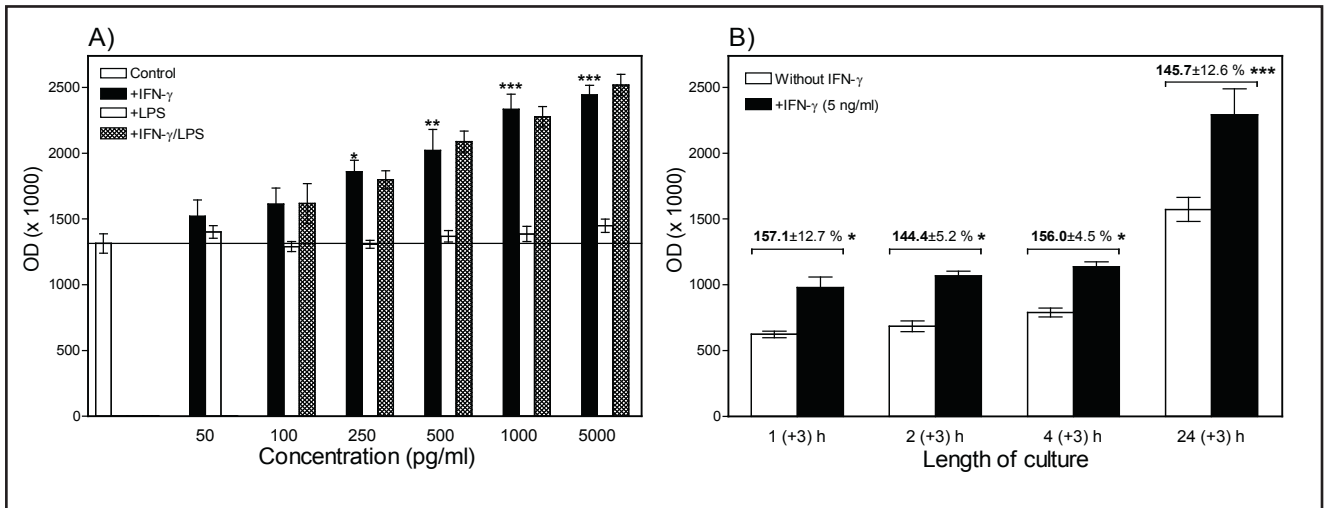
#### Suppressive effects of pyrimidine derivatives

In dependence on their structure (Jansa *et al.* 2014), selected pyrimidine derivatives (50  $\mu$ M) differentially interacted with the immune (IFN- $\gamma$ /LPS)-stimulated production of NO (Figure 5A). The group I compounds were devoid of any inhibitory effects. The group II and III pyrimidines inhibited NO production by approximately 50% and 80%, respectively ( $p < 0.001$ ).

Determined by the WST-1 assay, no compound had significant effect on formazan levels in supernatants of control (non-stimulated) cells. In contrast, the IFN- $\gamma$ /LPS-induced enhancement of formazan was significantly diminished ( $p < 0.0001$ ) by derivatives belonging to the pyrimidine groups II and III (Figure 5B), *i.e.* those which inhibited the NO production as well (Figure 5A). The formation of formazan dropped down to the values that were statistically indistinguishable from those detected in supernatants of control, IFN- $\gamma$ /LPS-untreated cells ( $F_{/4,23/} = 0.57$ ,  $p = 0.69$ ).

Accumulation of formazan which was determined by LDH assay (Figure 5C) remained uninfluenced by pyrimidine derivatives, no matter whether detected in supernatants of cells cultured in absence or presence of immune (IFN- $\gamma$ /LPS) stimuli (Figure 5C).





**Fig. 4.** Dependence of formazan production by mouse peritoneal cells (50,000/well) on concentrations of IFN- $\gamma$ , LPS and combination of both (A), and length of their action (B). Formazan was determined by WST-1 assay after 24 h (A) or indicated time intervals (B) following administration of stimulatory agents. Each bar is mean $\pm$ SEM. The data are representative of two identical experiments. Statistical significances: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

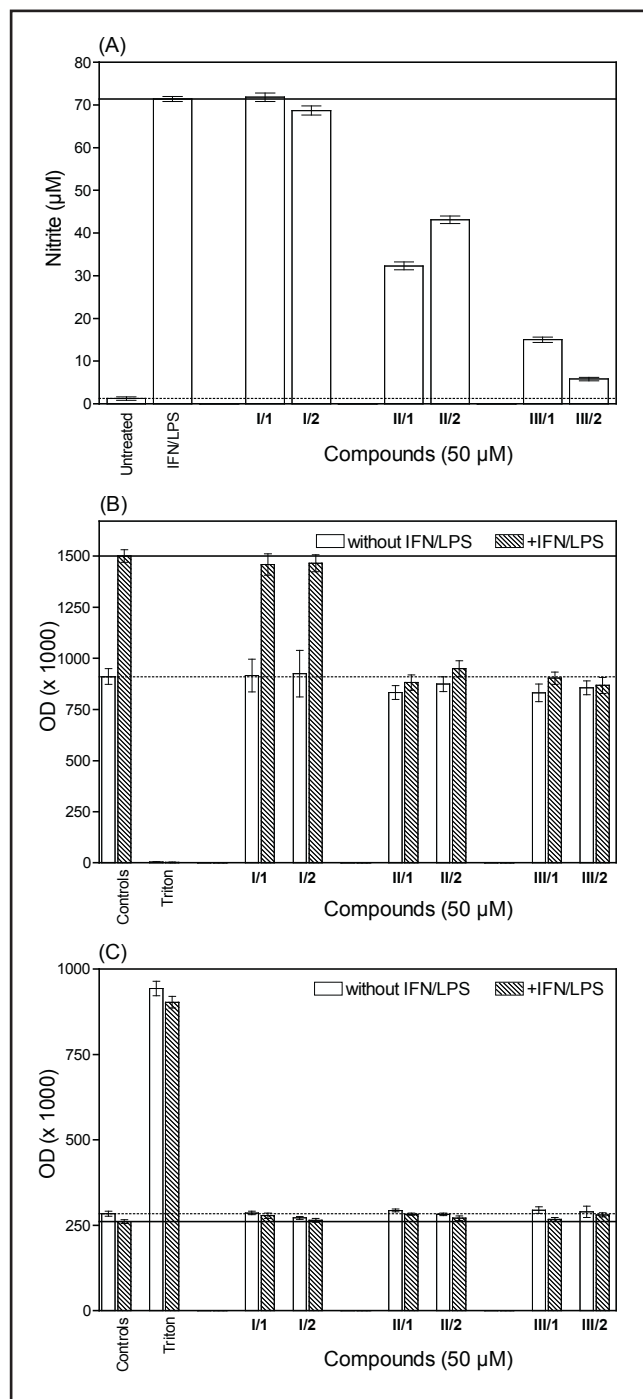
## DISCUSSION

We have found that the SDH-produced formazan, a product of the metabolic reduction of tetrazolium salt WST-1, is substantially enhanced upon the exposure of murine resident peritoneal cells to IFN- $\gamma$ . Interestingly, lymphocytes obtained from the spleen of mice have been found unresponsive to the action of IFN- $\gamma$ . Neither up-regulatory nor down-regulatory effects of LPS applied alone or in combination with IFN- $\gamma$  have been observed. The stimulatory effect of IFN- $\gamma$  on formazan production is due to the reactivity of macrophages represented here by adhered subpopulation of peritoneal cells. Since murine resident peritoneal macrophages (as well as pulmonary alveolar and tissue ones) are terminally differentiated non-dividing cells (Crume *et al.* 2007; Sodhi & Sethi 2004; van oud Alblas & van Furth 1979), participation of cell proliferation on the formazan enhancement can be ruled out. The results thus suggest that IFN- $\gamma$  may be considered as an activator of the macrophage respiratory complex II. This is in agreement with findings showing increased mitochondrial activity by 30-50% in IFN- $\gamma$  treated human lung cancer cell lines COR-L23 (Jabbar *et al.* 1989), and also in conformity with the protective effect of IFN against decreased formazan production induced by cytotoxic viruses (Berg *et al.* 1990). Alike, production of formazan resulting from the reduction of tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was stimulated by IL-3 in the bone marrow-derived cell line (Berridge & Tan 1993). However, these findings do not conform to data showing the opposite influence of cytokines on the activity of cell respiratory enzymes. Both respiratory complex I (NADH: ubiquinone oxidoreductase)

and complex II (SDH) were found suppressed upon the *in vitro* activation of murine macrophages with IFN- $\gamma$  or IFN- $\alpha/\beta$  in presence of LPS (Drapier & Hibbs 1988). The enzymes also were synergistically inhibited by IFN- $\gamma$  and TNF- $\alpha$  in rat smooth muscle cells though the effect was not accompanied by reduced cell viability (Geng *et al.* 1992). TNF- $\alpha$  on its own blocked electron transfer mediated by mitochondrial complexes I, II, and IV (ferrocytochrome c: oxygen oxidoreductase) in leukemic cells (Jia *et al.* 1996). The same as TNF- $\alpha$  also IL-1 $\alpha$  suppressed the respiratory chain complexes I and II in cardiomyocytes (Zell *et al.* 1997). Sources of these divergences are not clear. At least some of them might be accounted for by the use of different cytokines, different cell types and their origin and varying experimental conditions.

Mechanistically, stimulation of the respiratory complex II by IFN- $\gamma$  could be explained by IFN- $\gamma$  signalling. Binding of IFN- $\gamma$  to the type II IFN receptor initiates signalling through transphosphorylation and activation of the JAK1/JAK2 (Darnell *et al.* 1994). They mediate tyrosine (Y701) and serine (S727) phosphorylation of STAT1 leading to dimerization of STAT1 and its subsequent nuclear translocation, DNA binding, and stimulation of expression of IFN-responsive genes (Decker & Meinke 1997).

The activation of JAK/STAT pathway is usually very rapid and transient. Activated STAT molecules can be detected in the nucleus within minutes or a few hours (Wang *et al.* 2012). However, IFN- $\gamma$  signalling may differ in dependence on the type of cells (van Boxel-Dezaire & Stark 2007), the greatest response to IFN- $\gamma$  being expressed by monocytes (Waddell *et al.* 2010). These facts could at least partially explain our observation of remarkable and rapid onset of augmented SDH



**Fig. 5.** Effects of pyrimidine derivatives I/1 – III/2 (for specification see Materials and Methods) on production of NO (A) and supernatant concentration of formazan determined by WST-1 (B) or LDH (C) assays. Mouse peritoneal cells were grown in presence of IFN- $\gamma$  (5 ng/ml) plus LPS (0.1 ng/ml). Pyrimidines (50  $\mu$ M) were applied concomitantly with the immune stimuli. Triton was used to induce death of all cells. The effects were evaluated at the 24-h interval of culture. Each bar is mean  $\pm$  SEM. The data are representative of two identical experiments.

activity in macrophages on one site, whereas contrasting unresponsiveness of splenocytes to the action of IFN- $\gamma$  on the other one.

In addition to STAT1, also other members of STAT family, such as STAT3 and STAT5 are activated in

response to IFN- $\gamma$ , often in a cell type specific or maturation stage-specific (van Boxel-Dezaire & Stark 2007). Importantly, IFN- $\gamma$  and IFN- $\gamma$  receptor (Lambert *et al.* 2000) as well as STAT1, STAT2, STAT3, STAT5 and STAT6 (Meier & Larner 2014) are present in mitochondria. It has been suggested that maximal activities of complex I and II of electron transport chain depend mainly on STAT3 (Wegrzyn *et al.* 2009) though it obviously regulates cellular respiration in a transcriptional-independent manner (Tammineni *et al.* 2013). It cannot be excluded that it functions as a posttranslational modulator of mitochondrial respiratory complexes (Szczepanek *et al.* 2012). Anyhow, the JAK-STAT signalling pathways alone do not seem to be sufficient to explain all biological effects of IFN- $\gamma$  (Ramana *et al.* 2002; van Boxel-Dezaire & Stark 2007). It would be therefore premature to ascribe the IFN- $\gamma$ -induced complex II activity to the involvement of STAT3 solely. Further experiments are needed to elucidate mechanism(s) of the action.

Similar to the activity of SDH, also the activity of LDH is liable to regulation by various physiological mediators. For example, activators of cyclic AMP such as forskolin, cholera toxin and 1-methyl-3-isobutylxanthine induced the LDH activity in rat vascular smooth muscle cells (Marti *et al.* 1994). LDH messenger RNA was found stimulated by cytokines TNF- $\alpha$  and EGF (epidermal growth factor) in porcine testicular Sertoli cells (Boussouar & Benahmed 1999; Boussouar *et al.* 1999). The same cells showed enhanced expression of LDH messenger RNA and increased activity of LDH A4 isoform upon exposure to IL-1 $\alpha$  (Nehar *et al.* 1998). IFN- $\gamma$  suppressed the synthesis and activity of LDH stimulated by catecholamines isoproterenol and noradrenaline in rat glial cancer C6 cells (Passaquin *et al.* 1986). In variance to these reports, we have seen no changes in the activity of LDH in mouse macrophages stimulated with IFN- $\gamma$ , LPS or combination of IFN- $\gamma$  and LPS.

In order to assess possible impact of immune-triggered changes in activity of SDH on interpretation of cytotoxicity assays, selected pyrimidines have been used as model compounds. We have shown recently (Jansa *et al.* 2014) that they are the structure-dependent inhibitors of NO biosynthesis which are free of cytotoxic effects as determined by WST-1 assay in normal, *i.e.* immune-unstimulated murine peritoneal cells. Present additional experiments show that the pyrimidines diminish the IFN- $\gamma$ -enhanced production of formazan in this assay. The enhanced levels of formazan dropped down to the levels typical for spontaneous, *i.e.* constitutive production formazan in control cells. Neither the SDH-produced formazan in control cells nor the LDH-dependent formazan formation in both control and IFN- $\gamma$ -treated cells were affected by tested pyrimidines. Therefore, only seemingly could the reducing effects of pyrimidines on SDH-formed formazan under the conditions of IFN- $\gamma$  stimulation be considered as a sign of

cytotoxicity. The inhibitory potential has been found to be bound to those pyrimidines which possess the NO-inhibitory properties. The data thus suggest that tested pyrimidines inhibit transactivating pathways stimulated by IFN- $\gamma$  and implied in the up-regulation of both SDH and inducible NO synthase enzyme expression.

## CONCLUSION

The concentration of formazan determined by the WST-1 assay was found substantially enhanced in supernatants of murine macrophages cultured in presence of IFN- $\gamma$ . Under the same experimental conditions, IFN- $\gamma$  remained ineffective to influence formazan formation in the lactate dehydrogenase (LDH) assay. It is likely therefore that IFN- $\gamma$  activates the respiratory enzyme complex II (succinate dehydrogenase; SDH) and does not change the activity of LDH.

Both WST-1 and LDH assays are commonly used in screening for cytotoxic effects of compounds. The present findings indicate that immune status of cells may bear a significant impact upon the interpretation of these assays.

WST-1 assay showed that model pyrimidine derivatives diminished the IFN- $\gamma$ -enhanced concentration of formazan, decreasing it to the level of spontaneously formed formazan by control cells which remained unchanged by pyrimidines. The compounds did not affect the LDH-assayed accumulation of formazan in supernatants of both IFN- $\gamma$ -treated and control cells. It follows that these cannot be considered to possess cytotoxic activity. Mechanism(s) of their inhibitory action remain to be elucidated. The findings encourage further studies on possible interference of recently disclosed pyrimidines with IFN- $\gamma$  signalling pathways.

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