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DR HONG WAN (Orcid ID : 0000-0003-3794-5692)

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Induction of p53 in keratinocyte cultures treated with Behçet's patient sera

Sihem Houacine[1], Angray Kang[1], Eric Kenneth Parkinson[1], Hong Wan[1,*] and Farida Fortune[1,*]

[1]*Centre for Oral Immunobiology and Regenerative Medicine, Institute of Dentistry Barts and The London, School of Medicine and Dentistry, Queen Mary University of London, UK*

Running head: BD sera stimulates p53 expression.

To whom correspondence should be addressed:

*** Professor Farida Fortune CBE, BDS, MBBS, MRCP, FRCP, FDS RCSeng, FGDP, PhD and Dr. Hong Wan BDS, MSc, PhD**

Centre for Oral Immunobiology and Regenerative Medicine
Institute of Dentistry
Barts and The London, School of Medicine and Dentistry
Turner Street
Whitechapel
London E1 2AD

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Tel: 0044 (0) 20 7882 7139

Fax: 0044 (0) 20 7882 7137

E-mail: *h.wan@qmul.ac.uk*

ORCID iD: orcid.org/0000-0003-3794-5692

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Conflict of Interest

The authors declare no conflict of interest.

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Abstract

Background: Behçet's disease is a rare, multisystem vasculitis disease characterized by recurrent orogenital ulcerations with its etiology remained unclear. The transcription factor p53 has been reported to be upregulated in some autoimmune diseases, such as lupus erythematosus, dermatomyositis, and psoriasis. However, little is known about its alteration in Behçet's disease.

Methods: Keratinocyte cultures of both skin and oral origins were treated sera of 18 Behcet patients for 24 hours and analyzed by indirect immunofluorescence for p53 expression. The specificity of p53 expression was confirmed by siRNA mediated p53 knockdown and the serum IgG removal studies. The expression of p53 levels was quantitatively analyzed with ImageJ.

Results: It was shown that the expression of p53 is increased in skin and oral keratinocyte cell lines, in both the nucleus and cytoplasm of cells treated with patient sera compared to controls. Either p53 knockdown or IgG removal results in a reduction of p53 levels relative to cells treated with patient sera without p53 knockdown or IgG depletion.

Conclusions: This *in vitro* study provides the first evidence that Behçet's disease sera can induce the p53 expression in keratinocytes that may have implications in Behcet pathogenesis.

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1. Introduction

Behçet's disease (BD) is a rare, multisystem vasculitis disease of unknown cause and is characterized by recurrent orogenital ulcerations, skin lesions, musculoskeletal, gastrointestinal, ophthalmic, neurological and vascular involvement (1;2). It is thought that certain infectious factors (in particular Streptococcus Sanguis) can trigger the symptoms in genetically predisposed individuals (3). Some studies suggest that patients with BD were more susceptible to develop cancer, however, other studies report its association with lower incidence of cancer thus the association of BD with cancer is still a controversial issue (4;5). Currently, no curative solutions are available in the clinical treatment of BD. Nevertheless, treatment of BD aims to relieve symptoms, reduce tissue damage, resolve inflammation, eliminate recurrence and prevent life-threatening conditions (6). There continues to be an interest in trying to understand the underlying etiopathogenesis of BD.

p53 is a transcription factor predominantly located in the nucleus that regulates a host of molecular functions (review by Karen or someone similar). In response to stress signals such as DNA damage and various cellular insults, p53 is activated with an increased expression which in turn triggers different biological outputs, such as cell-cycle arrest, programmed cell death (apoptosis), modulation of autophagy or senescence (7;8). Overexpression of p53 has been reported in various diseases such as rheumatoid arthritis (RA) (9), discoid lupus erythematosus (DLE) (10), psoriasis (11), cutaneous lupus erythematosus (CLE) and dermatomyositis (12;13). However so far, little is known about the alteration of p53 in BD. Our recent study has shown that treatment of keratinocyte cell cultures with patient sera from pemphigus vulgaris (PV), an autoimmune blistering disease, evokes elevated p53 expression levels (14). Here in this present study, we investigated whether BD patient sera are capable of inducing enhanced p53 expression in keratinocyte cultures. Our results generated from this *in vitro* study indicate on exposure to BD sera there is induction of p53 in keratinocytes derived from both the skin and oral mucosa. This novel finding reveals important information on BD pathogenesis that could lead to p53-based therapeutic interventions.

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2. Materials and Methods

2.1 Cell lines and BD sera samples

Two different cell lines, *i.e.* NTERT immortalized skin keratinocytes and oral keratinocytes OKF6/TERT1 derived from floor of mouth, were used in this study and both are known to express wild type p53 (15). Cells were maintained in keratinocytes serum-free medium (KSFM) (17005402, Thermo Scientific) routinely and were grown in keratinocyte growth medium (KGM) containing three parts of DMEM Dulbecco's Modified Eagle Medium (DMEM) (12-604F, Lonza), one part of Ham's F12 medium (Lonza), 10% FCS, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin and 1% penicillin-streptomycin for experiment with BD sera treatment. They were kept at 37°C in a humidified atmosphere of 5% CO_2/95% air Normal control human serum was purchased (Sigma) and 18 sera from BD patients obtained from the London Behcet's Centre fully characterized (Table 1) with informed patient consent and ethical approval (Committee and reference number). The sera samples were preserved at -20 °C and were used to treat NTERT and OKF6/TERT1 cells, respectively. All sera were diluted in KGM at 40% except for the serum dosage experiments.

2.2 Antibodies

p53 mAb (DO-1) (ab1101, Abcam) and Alexa fluor 488 goat anti-mouse IgG were used, all in 1/150 dilution.

2.3 Immunofluorescence (IMF) staining

The general IMF staining procedures were followed throughout experiments to stain cells grown on coverslips in 24-well plates. At least five images were taken in the arbitrary regions for each sample and saved for image analysis later. All images were processed and analyzed with ImageJ, which is a Java-based image processing program developed by National Institutes of health.

2.4 siRNA transfection

The transfection procedures followed the protocol provided with DharmaFECT reagent (14). For p53 siRNA transfection, 2×10^5 cells were seeded in 6-well plate per well and were allowed to grow for 24 hours before transfection in OPTIMEM 1 (Thermo Scientific). The scrambled control and p53 specific siRNA were mixed with DharmaFECT reagent in OPTIMEM 1 in the hood and

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incubated for 10 minutes before adding into the culture medium at the final concentration of 80 nM. After 24 hours, cells were harvested and plated on coverslips in KSFM medium. Then, the medium was replaced with KGM and cells were allowed to grow overnight. The next day, the sera samples were prepared at 40% in KGM as described above before being added into each well and incubated for 24 hours prior to fixation and IMF staining.

2.5 IgG depletion

This experiment was performed twice. First**,** 100µl of Protein G Sepharose (ab193259) supplied as 50% slurry and 20% Ethanol was used for the IgG depletion from normal control and BD sera samples, respectively. In the second attempt, a similar protocol was performed using 200 µl of Protein G Sepharose.

3. Results

3.1 Enhanced p53 expression by BD sera in keratinocyte cultures

The first goal of this study was to determine whether the expression of p53 is increased in keratinocyte cultures treated with BD patient sera and this was performed in the NTERT cell line harboring wild type p53. Cells were seeded on coverslips in 24-well plate at confluent density for 1 day before being treated BD sera, alongside with control sera from normal healthy individuals, at the 40% concentration diluted in keratinocyte growth medium (KGM) and cells were fixed 24 hours later and were proceeded for indirect immunostaining for p53 with a mouse monoclonal antibody. In total, 16 patient serum samples were investigated compared to two normal control sera and pooled data in this analysis was shown in Figure 1. Enhanced p53 in both the nucleus and cytoplasm was indicated in cells treated with BD sera compared to control serum treated samples ($p<0.0001$, Figure 1A,B). The specificity of p53 expression and staining by the mouse monoclonal antibody was demonstrated by p53 specific knockdown with transient RNAi transfection with a marked decrease in the p53 staining by immunofluorescence (IMF) ($p<0.01$, Figure 1C). Some BD serum treated cells exhibited drastic morphological changes with remarkable p53 expression in both the nucleus and cytoplasm, suggesting some cytotoxic effect induced by the treatment of BD sera (data not shown).

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3.2 Enhanced p53 expression

Next, a dose-dependent experiment was performed with BD sera and in this case, cells were treated with a couple of BD serum samples at the final concentrations of 10% and 30%, respectively, alongside the normal control serum treated with cells as well as the cells without addition of sera. These results indicated a dose-dependent augmentation of p53 expression, though with some variations according to the individual serum samples (Figure 2). For instance, BD1772 exhibited an approximately 10-fold increase in p53 expression levels than BD3374 in a dose-dependent manner, with the latter showed no augmented p53 expression at all at the 10% concentration compared to the respected control ($p>0.05$, Figure 2). These data suggest further a specific effect in p53 elevation induced by BD sera. Interestingly, enhanced p53 levels also were associated with the addition of sera in the culture media regardless of disease condition (Figure 2).

3.3 Induction of p53 by BD sera was also detected in oral keratinocyte cells

BD patients primarily present ulcerations in the oral mucous membrane. We asked whether the same effect in the p53 induction also is detectable in oral keratinocytes with exposure to BD sera. To address this question, we treated an immortal oral keratinocyte line OKF6/TERT1 derived from the floor of mouth (gift from James Rheinwald (15)). Cells were seeded at the same densities and treated with BD and normal control sera at the 40% concentration for 24 hours, as described above, prior to IMF for p53. Ten BD serum samples were used to treat the OKF6/TERT1 cells alongside normal healthy control sera, however, seven of BD samples caused somewhat different morphology in OKF6/TERT1 cells, with diffuse p53 staining coupled with enlarged cell size (low panels Figure 3). Some of these cells exhibited condensed nuclear DAPI staining and concomitantly the faint p53 staining (arrows in Figure 3), whereas others showed the loss of nuclear staining by DAPI (arrowheads in Figure 3), suggesting cells had undergone programmed cell death or apoptosis. The other three BD serum treated cells displayed similar changes with elevated p53 expression as observed in NTERT cells and also showed a dose-dependent response in p53 expression (Figure 3A,C).

3.4 Induction of p53 by BD sera seemed to be dependent upon IgG

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Finally, we asked whether the induction of p53 by BD sera was primarily caused by IgG and to address this question, we depleted IgG with protein G prior to treating the NTERT cells alongside with control sera. Four BD sera samples were tested in this regard and showed a significant reduction of p53 in both the nucleus and cytoplasm, in particular in cells with a greater concentration of BD serum sample, *i.e.* 40%, compared to controls ($p<0.001$, Figure 4). These data suggest that the BD sera induced p53 expression is likely associated with IgGs existing in the patient sera.

4. Discussion

BD with unknown etiology is a disease affecting several organs but oral and genital ulcers are regarded as a hallmark of the disease and the first signs in most of the cases (16). Overexpression of p53 has been reported in some diseases such as in the synovium of RA (9), in the lesion skins of psoriasis (11) and the patients with DLE, CLE, and Dermatomyositis (10;13). Thus, the enhanced p53 expression seems to be associated with pathogenesis of several diseases. This study aimed to determine whether BD sera is capable of inducing p53 expression and thus, we performed IMF analysis for p53 expression in keratinocyte cultures treated with BD sera from a cohort of 18 patients with BD. Both skin-derived NTERT and oral-derived OKF6/TERT1 keratinocyte cell lines were used in the study. The levels of p53 expression and subcellular distribution in keratinocytes treated with control and BD sera for 24 hours were analyzed by IMF combined with quantitative image process. A significant increase of p53 was detected in cells treated with BD sera compared to controls, especially in nuclear p53, suggesting activation of p53. Similar findings were observed in both NTERT and OKF6/TERT1 keratinocyte cell lines. Nevertheless, the OKF6/TERT1 cells seemed to be more sensitive and showed drastic morphological changes in response to some BD sera samples with nuclear condensation or the loss of DAPI staining (*e.g*. BD2327, Figure 3A). Some BD sera induced the augmented p53 in both the nuclear and cytoplasmic, whereas others caused only pronounced cytoplasmic p53 expression. The effect of BD sera on p53 induction was demonstrated in a dose-dependent manner in both NTERT and OKF6/TERT1 cells. In addition, the specificity of p53 expression was verified by using p53 RNA interference which abrogates almost all the p53

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staining signals induced by sera (Figure 1C) in both control and BD serum treated cells, especially the cytoplasmic p53 in cells treated with BD sera. Enrichment of the p53 pathway has been reported in the peripheral blood cells of BD using the microRNA microarray analysis (17). Furthermore, an upregulation of the apoptotic genes, for example, BCL2-related, MCL-1, and BCL2L11 were found in the peripheral blood cells of BD patients in a study of gene expression profiling (18).

Finally, to address the effect of sera IgG on p53 induction, we removed IgG from BD and control sera by using protein G prior to treating the cells alongside the matched sera control without IgG depletion, and showed that IgG-depletion from BD sera resulted in a significant decrease of p53 expression compared with the matched BD sera without IgG removal, suggesting that IgG is likely responsible, at least in part, for the p53 induction in cells treated with patient sera.

The increased p53 expression in keratinocytes with BD sera exposure could be due to the high levels of inflammation in BD which triggers the induction of p53 or due to the monoclonal antibody drugs used in the treatment of BD. It has been reported that infliximab can cause induction of p53 (19;20). A study conducted by Mitoma et al. reported the role of infliximab in the upregulation of proteins, such as Bax, Bak, and p21$^{WAF1/CIP1}$, suggesting the activation of the p53 pathway (20). Overexpression of p53 was also found in psoriatic keratinocytes in patients treated with infliximab (19;21). In this present study, some clinical information was obtained, for example, the BD 3374 sera sample showed induction of p53, however, the patient was not under any drug treatment but had folliculitis at the time of taking the sample (Table 1). Thus, it would be necessary to evaluate the sera of patients with and without infliximab treatment.

In summary, this study provides the first evidence that BD sera are able to provoke p53 expression in keratinocyte cultures, in a dose-dependent manner, suggesting some pathological factors existing in the patient sera. In addition, the study suggests that the sera IgG could be a potential contributing factor in the induction of p53. These findings may have implications in the pathogenesis of BD. It is necessary to investigate the p53 expression in BD patient tissues.

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Figure Legends

Figure 1. Indirect immunofluorescence for p53 staining in NTERT cells treated with BD patient sera. (A) The representative images of p53 staining in NTERTs treated with healthy control (Norm Ct) or BD sera at the final concentration of 40% in keratinocyte growth medium for 24 hours. The insert was the technical negative control with the secondary antibody alone (Neg Ct). (B) Quantitation analysis of all samples indicated a significant increase of p53 expression, including both the nuclear (white asterisks) and cytoplasmic (black asterisk) p53 levels, in cells treated with BD sera compared to controls (16 BD cases included). (C) p53 knockdown almost completely abrogated the p53 signals by IMF (n=2). The same effect was also seen with BD sera (data not shown. (5 arbitrary fields per BD serum treated cells were analyzed to generate the mean value for each sample, two-tailed, unequal distribution Student's *t-test* was used to obtain the p values for comparison between the samples, *p<0.05;**p<0.01;****p < 0.0001).

Figure 2. Dose-dependent response in p53 expression in NTERT cells treated with normal control and BD sera, respectively. Top panels showed an example of p53 expression and its subcellular distribution in the cellular response to BD patient sera at 10% and 30% concentrations, respectively, alongside controls with normal sera or no addition of sera. Lower panels were the results of another example with BD sera that showed a more drastic effect in p53 expression, in particular in cells treated with 30% BD serum. The data were representative of at least two independent experiments. Grey asterisks were comparisons for cytoplasmic p53 between the samples and black asterisks were for the nuclear p53 comparisons between the samples. (n=5, two-tailed Student's *t-test*, **p <0.01;***p<0.001;****p < 0.0001).

Figure 3. Indirect immunofluorescence of p53 in oral keratinocyte OKF6/TERT1 cells treated with BD patient sera. (A) The representative images of p53 staining in cells treated with normal control (Norm Ct) and BD sera at a final concentration of 40% for 24 hours. Note that different p53 staining patterns and cell morphology were shown in cells treated with BD sera. Arrows indicated cells with low p53 expression whereas arrowheads indicated cells with negative DAPI nuclear staining in the absence or presence of p53 expression. (B) Quantitation of all samples (10 BD sera samples were included). (C) Dose-dependent augmentation of p53 expression and its subcellular distribution in OKF6/TERT1 cells treated with different concentrations of BD sera,

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alongside control sera. Grey asterisks were comparisons for cytoplasmic p53 between the samples. (n=5 fields per sample, *p<0.05; **p<0.01).

Figure 4. IgG removal from sera results in a reduction of p53 expression in both nucleus and cytoplasm in cells treated with BD sera, especially at the 40% serum concentration. NTERT cells were treated with BD sera with and without IgG depletion, at 40% (top panels) and 30% (lower panels) concentrations, respectively. A significant decrease in p53 expression levels was shown in cells treated with BD3374 at 40% (top panels) compared to controls. Data were a representative from two independent experiments (n=5 fields per sample, *p<0.05;**p<0.01;***p<0.001).

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Table 1. Patient characteristics used in the study

Patient	Sample ID	Age	Gender	Orogenital ulcerations
Patient-1	3726	42	Female	Yes
Patient-2	1772	25	Female	Yes
Patient-3	3374	35	Female	No
Patient-4	2327	27	Male	N/A
Patient-5	5388	25	Female	Yes
Patient-6	399	56	Male	No
Patient-7	639	39	Male	Yes (oral ulcers)
Patient-8	467	27	Male	No
Patient-9	1325	21	Female	No
Patient-10	6702	48	Male	N/A
Patient-11	8720	40	Female	Yes (oral ulcers)
Patient-12	5639	22	Female	Yes (oral ulcers)
Patient-13	379	23	Male	Yes (minimal oral ulceration)
Patient-14	8687	NA	Male	Yes (minimal oral ulceration)
Patient-15	1560	33	Male	Yes
Patient-16	3583	48	Male	No
Patient-17	4218	18	Male	No
Patient-18	391	23	Female	No

IgG depletion
-
Yes
Yes
-
-
-
-
-
-
Yes
Yes
-
-
-
-
-
-
-

A

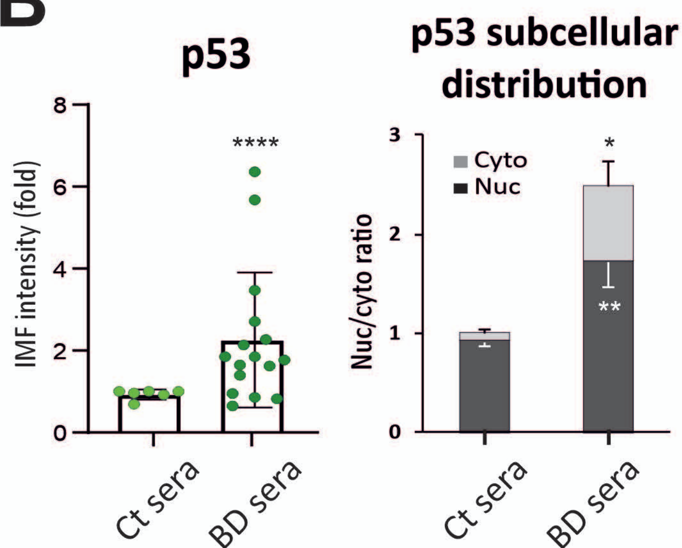
p53 | p53DAPI

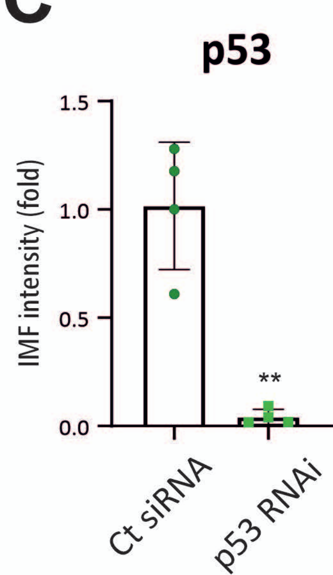
Norm Ct

Neg Ct

BD1772

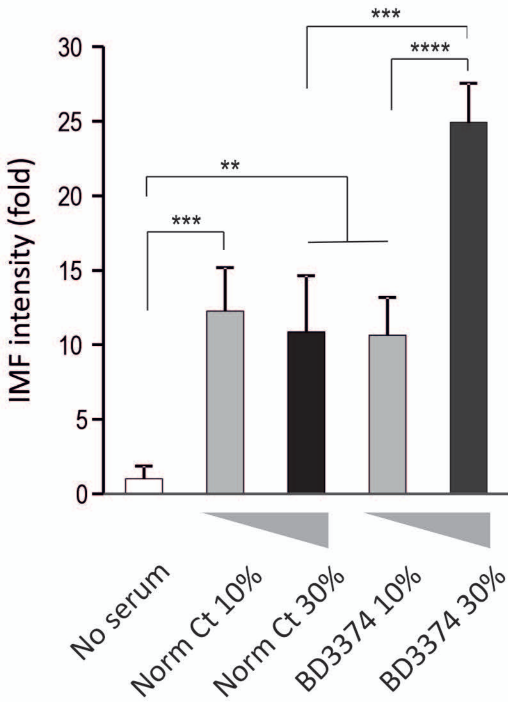
BD3374

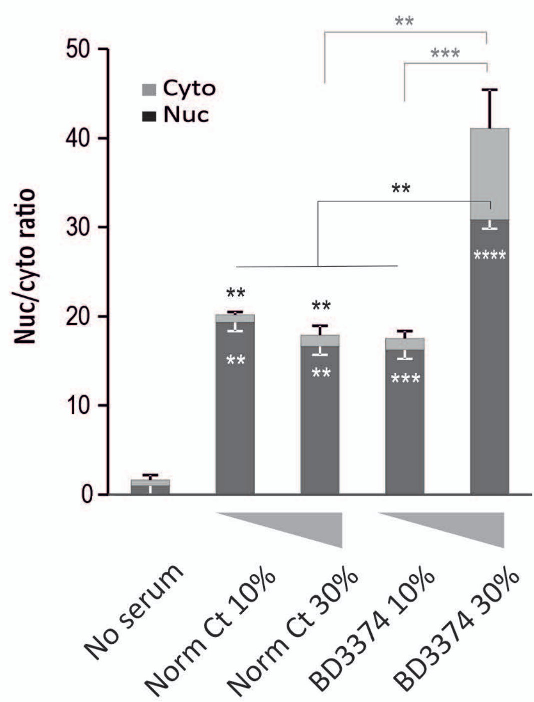


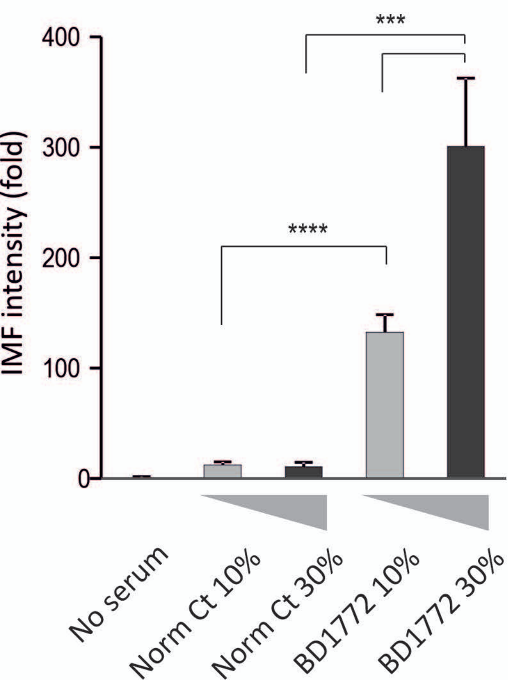


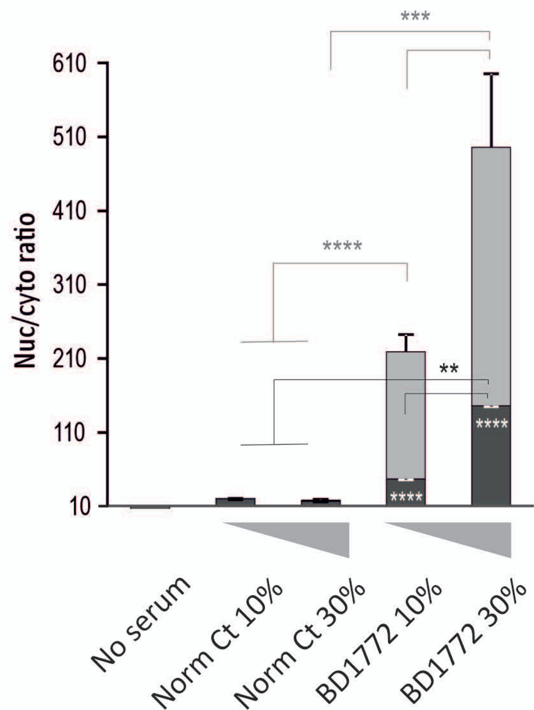
p53

p53 subcellular distribution









A

p53

p53DAPI

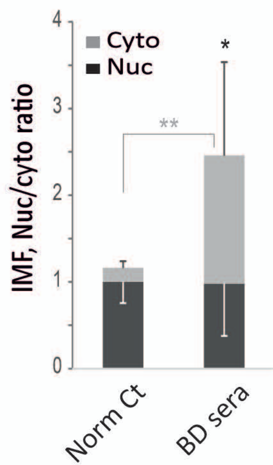
Norm Ct

BD1772

BD2327

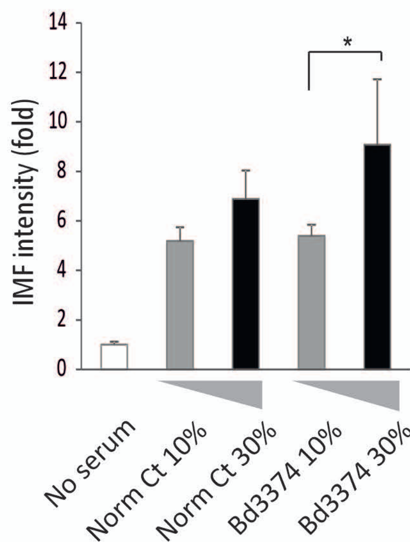
B

p53

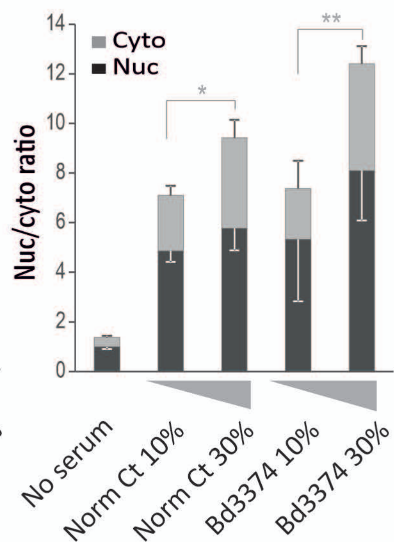


C

p53



p53 subcellular distribution



p53

p53 subcellular distribution