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Perturbation of neddylation-dependent NF-κB responses in the intestinal epithelium drives apoptosis and inhibits resolution of mucosal inflammation


ABSTRACT Recent work has revealed a central role for neddylation (the conjugation of a Nedd8 moiety to Cullin proteins) in the fine-tuning of the NF-κB response (via Cullin-1). In the present study, we investigated the contribution of Cullin-1 neddylation and NF-κB signaling to mucosal inflammatory responses in vitro and in vivo. Initial in vitro studies using cultured intestinal epithelial cells revealed that the neddylation inhibitor MLN4924 prominently induces the deneddylation of Cullin-1. Parallel Western blot, luciferase reporter, and gene target assays identified MLN4924 as a potent inhibitor of intestinal epithelial NF-κB. Subsequent studies revealed that MLN4924 potently induces epithelial apoptosis but only in the presence of additional inflammatory stimuli. In vivo administration of MLN4924 (3 mg/kg per day) in a TNBS-induced colitis model significantly accentuated disease severity. Indeed, MLN4924 resulted in worsened clinical scores and increased mortality early in the inflammatory response. Histologic analysis of the colon revealed that neddylation inhibition results in increased tissue damage and significantly increased mucosal apoptosis as determined by TUNEL and cleaved caspase-3 staining, which was particularly prominent within the epithelium. Extensions of these studies revealed that ongoing inflammation is associated with significant loss of deneddylase-1 (SENP8) expression. These studies reveal that intact Cullin-1 neddylation is central to resolution of acute inflammation.

INTRODUCTION Posttranslational protein modifications (PPMs) play an important role in the regulation of protein function, allowing for rapid responses to external stimuli (Song et al., 2010). One of these PPMs, neddylation—

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enzyme (NAE; also called UBA3-APPB1-E1-ligase; Wada et al., 1998; Mendoza et al., 2003; Huang et al., 2004). Subsequently the Nedd8 moiety is transferred to its E2-ligase (Jones et al., 2002) and then the target Cullin-E3-ligase protein complex, thereby activating it (Parry and Estelle, 2004). Free Nedd8 can be generated through cleavage of conjugated Nedd8 from the Cullin-E3-ligase, a process known as deneddylation. This process depends, at least in part, on the COP9 signalosome, can be positively influenced by commensal bacteria (Kumar et al., 2007; Jones et al., 2015) and extracellular adenosine (Khoury et al., 2007), and offers a potentially protective mechanism during inflammatory processes. In addition, loss of the isopeptidase sentrin-specific protease 8 (SENP8)/deneddylase-1 leads to a loss of neddylatory function and an inability to activate NF-κB (Ehrentraut et al., 2013).

Studies of neddylation and Cullin pathways in vivo have been hampered by the embryonic lethality of gene-targeted mice (Tateishi et al., 2000). Recent advances in the development of small-molecule therapeutics identified the adenosine monophosphate (AMP) homologue MLN4924 (Soucy et al., 2009). This compound disrupts the Cullin-E3-RING-ligase complex neddylation by inhibiting NAE. Given our previous observations that adenosine deneddylates Cullin proteins during ongoing inflammation (Khoury et al., 2007), we investigated the effect of MLN4924 on inflammatory responses and demonstrated that administration of this compound inhibits acute lipopolysaccharide (LPS)-induced endotoxemic shock (Ehrentraut et al., 2013). Other studies showed that low-dose MLN4924 activated HIF through Cullin-2 (Angus, 2011; Curtis et al., 2015). Here we sought to investigate the contribution of protein neddylation to mucosal inflammation using cultured epithelial cells and experimental colitis in mice as model systems.

RESULTS
Cytokine-induced Cullin-1 neddylation is abrogated by MLN4924

In the present study, we examined how neddylation affects epithelial NF-κB responses and mucosal inflammation endpoints. As shown in Figure 1A, Caco-2 intestinal epithelial cell exposure to tumor necrosis factor-α (TNF-α; 10 ng/ml, 1 h) induced p65 nuclear translocation. Coincubation of cells with the NAE inhibitor MLN4924 inhibited both p65 nuclear translocation and Cullin-1 neddylation in a concentration-dependent manner. At doses as low as 100 nM MLN4924, the neddylation fraction of Cull-1 was significantly decreased (p < 0.05). Parallel studies using NF-κB reporter assays revealed concentration-dependent inhibition of NF-κB activity with MLN4924, with >60% loss of activity at 3 μM MLN4924 (Figure 1B). When Caco-2 and T84 intestinal epithelial cells were exposed to the combination of MLN4924 (3 μM) and TNF-α/interleukin-1β (IL-1β; 10 ng/ml each), we observed a 50–70% decrease in the induction of the NF-κB target genes IL-8 and ICAM-1 (Figure 1C; p < 0.025). These results indicate that MLN4924 is a potent NF-κB inhibitor and that loss of Cul-1 neddylation significantly inhibits NF-κB target gene induction.

Epithelial barrier function and neddylation

One of the hallmarks of mucosal diseases, including inflammatory bowel disease (IBD), is epithelial barrier dysfunction (Koch and Nusrat, 2012), allowing for translocation of luminal contents into the serosa. Here we examined the influence of neddylation on epithelial barrier function in the presence and absence of inflammatory stimuli. Epithelial barrier function has been widely modeled in vitro through measurement of transepithelial electrical resistance (TER).

For these purposes, T84 intestinal epithelial cells were cultured on polycarbonate inserts and grown to confluence (>1000 Ω.cm²). Cells were exposed to medium alone (control), cytokin (10 ng/ml each of TNF-α, IL-1β, and interferon-γ), MLN4924 (1 μM) alone, or the combination of cytokin and MLN4924. As shown in Figure 2A, exposure of confluent epithelia to MLN4924 alone did not influence baseline epithelial barrier compared with medium alone (p = 0.54), suggesting that neddylation per se is not necessary to maintain epithelial barrier function. Treatment of epithelia with cytokin led to a significant (p < 0.05) decrease in epithelial resistance over 24 h, indicative of a loss of tight junctional integrity (Figure 2A), which has been previously demonstrated (Brewer et al., 2003). This cytokine-dependent decrease in barrier was markedly enhanced in combination with MLN4924 (Figure 2A; p < 0.01 by analysis of variance [ANOVA]), indicated by an earlier and more severe reduction in TER measurements.

TER measurements reflect changes in electrical conductivity from both transcellular and paracellular paths. To verify whether the observed changes were attributable to the paracellular pathway, we performed paracellular flux assays using 3-kDa fluorescein isothiocyanate (FITC)-dextran as a tracer. As shown in Figure 2B, similar to TER measurements, MLN4924 alone did not increase paracellular flux compared with control (p = 0.10), whereas cytokin treatment increased paracellular flux by a small (10%) but significant amount (p < 0.05). The combination of MLN4924 and cytokin, however, increased transepithelial flux by nearly 50-fold (p < 0.01) compared with other treatment groups, clearly indicating that the loss of neddylation in combination with inflammatory stimuli results in a marked loss of tight junction integrity.

Previous studies showed that the disruption of epithelial junctions in response to inflammatory cytokines is related at least in part
to an increase in apoptosis through the activation of caspase-3 (Nava et al., 2010). Once activated, caspase-3 initiates the nonreversible apoptotic cascade leading to DNA fragmentation and ultimately cell death (Porter and Janicke, 1999). To determine whether increased apoptosis was responsible for our observed neddylation-dependent loss of barrier, we characterized the influence of the combination of cytokine activation and MLN4924 on caspase-3 activity. As depicted in Figure 2C, MLN4924 alone did not significantly influence caspase-3 activity (p = 0.12). Although cytomix alone trended toward increased caspase-3, the combination of neddylation inhibition and cytomix increased caspase-3 activity by nearly ninefold compared with control and MLN4924 alone (p < 0.01). To define the functional significance of this observation, we used a general caspase inhibitor peptide to block caspase-3 activation. As shown in Figure 3A, the decrease in TER caused by cytomix in combination with MLN4924 was significantly attenuated by the caspase inhibitor peptide compared with cells treated with a negative control peptide over a 24-h period (p < 0.05 by ANOVA). The earliest time point of animal death was 2 d into the trial period. Treatment with MLN4924 alone (3 mg/kg per day) did not alter this ratio, and all of the animals survived the 3-d trial period (n = 5). Daily subcutaneous injections of MLN4924 combined with TNBS significantly increased mortality (60% of animals by day 3.5, n = 10, p < 0.025 compared with vehicle treatment; Figure 4A).

Colon shortening, a hallmark feature of murine colitis (Karhausen et al., 2004), was not different between vehicle and MLN4924-alone exposures (Figure 4B). Colitis induced with TNBS showed a nonsignificant trend toward shorter colons at the time of killing, which was consistent with previous data for this model (Robinson et al., 2008). However, animals receiving repetitive doses of MLN4924 along with the induction of colitis showed a significant reduction of colon length compared with their littermates receiving only TNBS (Figure 4B).

TNBS colitis has been shown to lead to apoptotic cell death (Crespo et al., 2012; Hjerpe et al., 2012). TNBS-induced inflammatory response was characterized by a loss of crypt architecture and infiltration of large numbers of inflammatory cells with mucosal and submucosal injury (Figure 4C, hematoxylin and eosin staining). MLN4924 treatment alone resulted in no observable change to colonic architecture compared with vehicle control. The combined use of MLN4924 and TNBS, however, significantly increased tissue destruction, leading to total loss of crypt structure, massive inflammatory cell infiltration, and transmural mucosal denudation (Figure 4C, bottom left). As a result, the tissue injury index in mice receiving MLN4924 with TNBS was significantly increased compared with those receiving TNBS alone (Figure 4F).

Neddylation and intestinal inflammation in vivo

Having defined the importance of neddylation for epithelial barrier integrity in vitro, we extended these results to define the relative importance of neddylation in colonic inflammation in vivo, using a murine 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis model. This is a model characterized by disruption of the epithelial barrier in vivo (Karhausen et al., 2004). After TNBS instillation, body weight was monitored twice per day. In accordance with previous observations, TNBS treatment led to increased mortality (33% after 3 d, n = 9, compared with 0% death in control group, n = 5, treated with ethanol only; Figure 4A). The earliest time point of animal death was 2 d into the trial period. Treatment with MLN4924 alone (3 mg/kg per day) did not alter this ratio, and all of the animals survived the 3-d trial period (n = 5). Daily subcutaneous injections of MLN4924 combined with TNBS significantly increased mortality (60% of animals by day 3.5, n = 10, p < 0.025 compared with vehicle treatment; Figure 4A).
In addition, colonic tissue of animals treated with only MLN4924 showed no increase in the amount of fragmented DNA (a sign of apoptotic cell death) compared with vehicle-treated controls (Figure 4D; terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL] staining, top right and left). TNBS-induced colitis increased the detectable amount of fragmented DNA, indicative of some degree of cell death. This was increased in the colonic epithelium of animals receiving both TNBS and MLN4924 (Figure 4D, bottom). The activation of caspase-3 is another keystone along the apoptotic death pathway. In our study, MLN4924 alone did not alter the degree of cell death. This was increased in the colonic epithelium of animals undergoing TNBS colitis while concomitantly being dosed with MLN4924 (approxi-mately sixfold compared with normalized control, p < 0.01; Figure 4G).

We previously showed that human deeneddylase-1 (SENP8) is crucial for enabling NF-κB-mediated inflammation (Ehrentraut et al., 2013). Whether SENP8 itself is regulated during chronic inflammation is unknown. For this purpose, we investigated the expression of SENP8 in our model systems (cultured epithelia, murine tissue), as well as human tissue from healthy and IBD subjects. As shown in Figure 5, there was a striking similarity between the three models that revealed a correlation between disease and the loss of SENP8 mRNA expression. Exposure of T84 cells to cytomix (24 h) resulted in a nearly 70% decrease in SENP8 mRNA expression (p < 0.01; Figure 5A). Epithelial isolates from animals undergoing TNBS colitis at days 1 and 3 after induction were examined and compared with ethanol-only controls. TNBS-colitic animals tended to express less SENP8 mRNA compared with healthy controls (Figure 5B) and day 3 after colitis induction (p < 0.05, n = 5 per group; Figure 5B). The same response was observed in human tissue samples from patients with active IBD. Regardless of the type of colitis (i.e., ulcerative colitis vs. Crohn’s disease), mRNA levels of SENP8 were significantly lower than in samples from healthy individuals (p < 0.001; four nonactive controls, 19 individual samples per disease cohort; Figure 5C). Collectively these results suggest that intact neddylation is disease protective and that loss of SENP8 expression correlates with the development of mucosal inflammatory disease in both mice and humans.

**DISCUSSION**

Posttranslational modifications of signaling proteins are critical to productive inflammatory responses and resolution of disease (Ehrentraut and Colgan, 2012). NF-κB is the quintessential signaling hub during acute inflammation, and its regulation is fine-tuned by multiple posttranslational modifications, including neddylation (Amir et al., 2002). Cullin proteins, as components of ubiquitin E3 ligases, are neddylated for the polyubiquitination of effectors (e.g., IκB). This neddylation response is regulated, in part, by the denedylation of SENP8 as a mechanism to control E3 ligase activity. An intact Cullin-3-IκB neddylation process is necessary for eliciting a coordinated inflammatory response to external inflammatory stimuli such as LPS (Ehrentraut et al., 2013), and this response is directly linked to the functional expression of human deeneddylase-1/SENP8. We demonstrate here that pharmacological targeting of neddylation using the AMP homologue MLN4924 potently blocks NF-κB activation/signaling and through these actions functions as a proapoptotic sensitizer (Figure 6).

The present studies revealed that MLN4924 is an effective (EC50 ~ 1 μM) Cul-1 deneddylating agent and equally potent inhibitor of NF-κB in intestinal epithelia. Presumably through its actions on Cul-1 activity and subsequent inhibition of E3 ubiquitin ligase activity, MLN4924 appears to function as a “priming” agent of the inflammatory response. Consistent with this premise, in vitro or in vivo exposure to MLN4924 alone resulted in surprisingly little activity within the mucosa. By stark contrast, the addition of activators of
FIGURE 4: Neddylation inhibition increases disease severity in a TNBS model of colitis. (A) Treatment of C57BL/6 mice treated with 2.5% TNBS and MLN4924 s.c. at 3 mg/kg per day shows decreased survival in mice receiving TNBS plus MLN4924 (29 total mice). (B) Decreased colonic length in mice receiving TNBS plus MLN4924 compared with TNBS alone. (C) Histological colonic sections of mice treated with or without TNBS and with or without MLN4924. (D) TUNEL staining in mouse colon sections from TNBS plus MLN4924 experiment. (E) Cleaved caspase 3 IF staining in mouse colon sections from TNBS plus MLN4924 experiment. (F) Analysis of tissue injury index in mice receiving a combination of TNBS plus MLN4924 in comparison to TNBS alone. (G) Increased IL-1 and IL-6 protein expression as detected by MesoScale in mice receiving TNBS plus MLN4924 compared with TNBS alone.

In intestinal epithelia, MLN4924 more potently inhibits Cul-2 than Cul-1. Our previous studies, in fact, revealed EC_{50} ≈ 5 nM for MLN4924 actions on Cul-2 and HIF stabilization (Curtis et al., 2015). In these studies, lower concentrations of MLN4924 (0.1 mg/kg, compared with 3 mg/kg used in the present studies) activated HIF in vivo and were protective for dextran sulfate sodium (DSS) colitis at multiple levels. There are distinct differences in our findings here, that is, aggravation of inflammatory tissue damage after MLN4924 plus TNBS–induced inflammatory disease and previously reported findings from our group in DSS colitis. These differences may reflect the different pathogenic mechanisms of both colitis models. DSS colitis is believed to occur independent of adaptive immune cells, whereas TNBS colitis is directly T-cell dependent (Neurath et al., 1996). MLN4924 was initially discovered for the treatment of NF-κB–dependent B-cell lymphoma (Milhollen et al., 2010). Hence use of MLN4924 in a disease model dependent on adaptive immune cells might explain the observed differences. Together these findings suggest that both in vitro and in vivo, Cul-2 responses are significantly more sensitive to MLN4924.

Given the central role of NF-κB in inflammation, it is not surprising that numerous studies have revealed that inhibition of NF-κB is antiinflammatory (Kanarek and Ben-Neriah, 2012). The intestinal mucosa—specifically, epithelial cells—appears to be somewhat unique in this regard (Karrasch and Jobin, 2008). For example, genetic deletion of NF-κB components (e.g., Ikkβ) within the intestinal epithelium results in significantly exacerbated pathogen-induced intestinal inflammation (Zaph et al., 2007). These studies revealed increased apoptotic responses with the loss of NF-κB signaling, resulting in a loss of epithelial barrier and ultimately septicemia. Our studies here indicate similar results with targeting neddylation in vivo and that as a preapoptotic sensitizer, the combination of deneddylation

In the presence of inflammatory stimuli significantly enhances the inflammatory response. It is noteworthy that throughout these studies, the inhibition of neddylation using MLN4924 had little to no detectable influence on basal epithelial function (i.e., in the absence of additional inflammatory stimuli). Barrier function, for example, was not changed by the inhibition of neddylation responses, even at relatively high concentrations of MLN4924. At multiple levels, these studies revealed a primed inflammatory response that correlated with the loss of neddylation and diminished NF-κB activity in vivo. Direct inhibition of NF-κB via Bay 11-7085 in conjunction with inflammatory stimuli partially recapitulated the reduction in TER measurements and thus barrier function observed with MLN4924.

A central component of the active deneddylation response is SENP8, an isopeptidase capable of directly deneddylylating Cullin

the NF-κB pathway profoundly enhanced the inflammatory response. Other model systems show similar actions of MLN4924. For example, studies of T-cell activation revealed that whereas MLN4924 alone does not activate T-cells, neddylation inhibition significantly lowers the threshold for anti-CD3–stimulated cytokine production (Friend et al., 2013). Other studies in T-cells show that MLN4924 may have neddylation targets beyond the Cullins, including proteins in the Ras/Erk pathway and other adaptor proteins such as Shc (Jin et al., 2013). Godbersen et al. (2015) also showed that targeting neddylation with MLN4924 abrogates NF-κB activation in leukemic B-cells and, in the process, regulates a diverse set of target genes. Thus the "priming" activity elicited by MLN4924 within the mucosa likely represents a complex response to neddylation targets beyond that of Cullins.
proteins (Mendoza et al., 2003; Wu et al., 2003), offering a cleavage pathway beyond the COP9 signalosome (Lyapina et al., 2001; Cope and Deshaies, 2003). It was shown, for example, that knockdown of SENP8 prevented LPS-induced NF-κB activation and systemic cytokine release (Ehrentraut et al., 2013). We extended these results to define the expression of SENP8 in murine and human colitis. Across each model tested, including human IBD tissue, inflammation was associated with a loss of SENP8 expression. Such observations suggest that down-regulation of SENP8 in murine/human colitis serves as a compensatory mechanism to quench the ongoing inflammatory response. Mechanisms of such regulation await further studies.

In conclusion, we demonstrate the contribution of epithelial NF-κB and Cul-1 neddylation to inflammatory responses in the intestinal mucosa. In particular, these studies identify MLN4924 as an inhibitor of Cul-1 neddylation, leading to loss of NF-κB signaling. These findings support a role for Cul-1 deneddylation as an apoptotic presensitizer during inflammation, which in turn enhances intestinal inflammatory responses.

MATERIALS AND METHODS

Cell culture

Human T84 and Caco-2 intestinal epithelial cells were cultured in 95% air with 5% CO2 at 37°C in DMEM and DMEM:F12, respectively, supplemented with 10% calf serum (Fisher Scientific, Waltham, MA) and penicillin/streptomycin (100 U/ml, 100 μg/ml; Invitrogen, Carlsbad, CA).

Transcriptional analysis

TRizol reagent (Invitrogen) was used to isolate RNA from Caco-2 or T84 cells. cDNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). PCR analysis was performed using SYBR Green (Applied Biosystems, Carlsbad, CA) and the following primer sequences: IL-8, forward 5′-CTGGCGGGTGGCTCTCTGG-3′, reverse 5′-CCTTGGCACAATCGACCTT-3′; ICAM-1, forward, 5′-ATGCCAGACATCTGTGTCC-3′, reverse 5′-GGGGTCTCTCTCTATGCCCAACA-3′; and β-actin, forward, 5′-GGAGAAAAATCTGGACCAAC-3′, reverse, 5′-AGAGGGCTACAGGGATAGCA-3′. Each experiment was performed in triplicate.

Western blot analysis

The NE-PER extraction kit was used to prepare nuclear and cytoplasmic lysates from Caco-2 cells per the manufacturer’s instructions (Thermo Scientific, Waltham, MA). Western blotting of these lysates was performed using Cul-1 Rb polyclonal antibody (pAb; Invitrogen), p65 Rb pAb (Cell Signaling, Danvers, MA), TATA-binding protein (TATA-BP) Ms monocolonal antibody (mAb; Abcam, Cambridge, United Kingdom), and human β-actin Rb pAb (Abcam). Each experiment was performed in triplicate.

Luciferase assays

An NF-κB luciferase reporter plasmid (500 ng; Ehrentraut et al., 2013) and a Renilla reporter plasmid (1 ng) were transfected into subconfluent Caco-2 cells using Lipofectamine LTX Reagent (Thermo Scientific). Luciferase activity was determined at 16 h using Promega dual luciferase reagents (Promega, Madison, WI), and luminescence was determined using the GloMax-Multi plate reader (Promega). Each experiment was performed in triplicate.

Barrier integrity, permeability assays, and apoptosis assays

T84 cells were plated on 0.33-cm2, 0.4-μm permeable polyester inserts (Corning, Corning, NY). TER was measured using the EVOM2 voltohmmeter (World Precision Instruments, Sarasota, FL) to monitor...
barrier formation after treatment of confluent T84 monolayers with MLN4924 (1 μM; Millennium Pharmaceuticals, Cambridge, MA), cytomix (10 ng/μl each of TNF-α, IL-1β, and IFN-γ; eBioscience, San Diego, CA), and Bay 11-7085 (30 μM; Tocris Bioscience, Minneapolis, MN).

Paracellular permeability was assayed using FITC-dextran flux assay described previously (Furuta et al., 2001) on T84 monolayers with MLN4924 (1 μM), cytomix, or a combination of both MLN4924 and cytomix.

To detect caspase-3/7 activity, the Caspase-Glo 3/7 Assay (Promega) was used according to the manufacturer’s instructions. Briefly, 10,000 T84 intestinal epithelial cells per well were plated in 96-well plates and treated with 1 μM MLN4924, cytomix, 30 μM Bay 11-7085, or a combination of either MLN4924 or Bay 11-7085 with cytomeg for 24 h. After 24 h, 100 μl of the Caspase-Glo 3/7 reagent was added to each well, and the luminescence was measured using the GloMax-Multi plate reader (Promega).

To inhibit caspase activation, a general caspase inhibitor peptide (Z-VA-D-FMK; 30 μM) or a caspase-3 inhibitor peptide (Z-DEVD-FMK; 30 μM) was given as a 30-min pretreatment to T84 cells plated on permeable polyester inserts before treatment with cytomeg plus MLN4924 (1 μM; BD PharMingen, San Diego, CA) or cytomeg plus Bay 11-7085 (30 μM). A negative control peptide (Z-FA-FMK; 30 μM) was used on additional inserts (BD PharMingen).

**Animals**

Wild-type C57BL/6 mice (Mus musculus) were obtained from Jackson Laboratories (Bar Harbor, ME). All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Colorado. TNBS colitis was induced as previously described (Louis et al., 2008). Animals were administered either MLN4924 (3 mg/kg) or cyclodextrin (Sigma-Aldrich, St. Louis, MO) via subcutaneous (s.c.) injection at day 1 of TNBS exposure, and this was continued daily. The groups were as follows: ethane, severe), extent of injury (0–3: none, mucosal, mucosal and submucosal, transmural), and crypt damage (0–4: none, basal one-third damaged, basal two-thirds damaged, only surface epithelium intact, entire crypt and epithelium lost). The score of each parameter was multiplied by a factor reflecting the percentage of tissue involvement (<1: 0–25%; ≥2: 26–50%; ≥3: 51–75%; ≥4: 76–100%), and all numbers were summed. Maximum possible score was 40.

**Human tissue**

Deidentified human intestinal tissue cDNA was obtained from the TissueScan Crohn’s/ColitisII array (OriGene Technologies, Rockville, MD). Complete patient/sample characteristics can be accessed from the supplier’s website (www.origene.com).

**Statistical analysis**

Data are expressed as mean values ± SEM. Data were analyzed with Student’s t test between two groups or ANOVA coupled with post hoc Bonferroni test for multiple pairwise comparisons. p < 0.05 was considered to be statistically significant.

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