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10.1515/amb-2015-0002

NALP SIGNALLING IS REQUIRED IN SERTOLI CELLS FOR TIGHT-JUNCTION PROTEIN INTERACTION

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Summary. The present study aims to investigate the NALP3 system and its influence on occludin in Sertoli cells, utilising primary murine cells and adult Sertoli cell line as models. Its main goals are the Sertoli cell biology with possible implications on male reproductive functions. Primary and adult Sertoli cells were transfected with NAPL3 siRNA and treated with NOD1 (ie-DAP) and NOD2 (MDP) receptor ligands. There was positive occludin expression levels on transcript (RT-qPCR) and protein (FCS and Immunofluorescence) levels for both cell types. The innate immunity and tight-junction pathways integration serve a protective role for both testis immune barrier and spermatogenesis compartmentalisation maintained by the very same barrier. This integration also points the way for mechanistic research of the disturbances inflicted during an inflammatory response in testis niche.

Key words: *Sertoli cells, tight-junctions, occludin, NALP3*

INTRODUCTION

Sertoli cells sustain blood-testis barrier forming tight-junctions (TJ) and other structures responsible for its selective permeability. Claudins [5] and occludin [6] were the first TJ integral membrane proteins identified. The normal function of Sertoli cells is tightly connected to the reproductive status of the male. Innate immunity resembles an ancient signalling system that deeply impacts the fundamental functions of the cells, and is currently becoming evident as being involved in cell fate, cell death, apoptosis and autophagy, as well as antigen presentation. The system of NLRP receptors and their downstream targets and NALP3 part of the inflammasome is currently under study and is being implicated in many autoimmune disease in men [1, 9]. Currently only a few studies have been conducted mostly with a scope on the role and abundance of TLRs in male reproductive system. The present study aims to investigate the NALP3 system and its influence on occludin (TJ) in Sertoli cells, utilising primary murine cells and adult Sertoli cell line as models. Its main goals are to validate if this system is present and operational,

and to look for possible impacts of its actions and perturbations on the Sertoli cell biology with possible implications on male reproductive functions.

MATERIALS AND METHODS

Reagents: The minimal bioactive peptidoglycan motif common to all bacteria and NALP3 activator conjugated with FITC dye muramyl dipeptide (MDP) as well as γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) – a dipeptide present in the peptidoglycan (PGN) of all Gram-negative and certain Gram-positive bacteria, both were purchased from Invivogen.

Primary Sertoli cell isolation and cell cultures: BALB/c mice (n = 80) were obtained (Slivnitca National Animal Facility, Bulgaria) and kept in accordance with the National Animal Care and Use of Laboratory Animals Standards. Seminiferous tubules from pre-pubertal (15-day-old) male animals were prepared, following the methods described by [3]. After removal of tunica albuginea, and PBS wash, a successive enzymatic digestion follows, using 0.1% collagenase, 0.1% hyaluronidase and 0.25% tripsin, and followed by centrifugation steps for washing and sedimenting the cells to remove the interstitial tissue. The interstitial contamination of the tubules is avoided by using a preparative microscope, and an additional hypotonic shock for depleting the germ cells 2 days after the initial plating of the cells. The primary Sertoli cells were cultured in BSA free, DMEM:F10 media (Sigma), supplemented with insulin, transferrin and pituitary gland extract. At the fourth day of culture, the primary cell monolayers covering artificial extracellular matrix (ECM, Sigma) were treated for 24h with compounds ie-DAP (4 μg/ml) and MDP (20 μg/ml). The established Sertoli cell line 15P-1 (ATCC), derived from 6 months-old adult male transgenic mice and maintained at 320 C in DMEM media was used. Similar experiments using innate immunity ligands induction were carried on.

Mouse NALP3 siRNA transfection and knockdown: The gene silencing experiments were carried out using HiPerfect transfection reagent (Qiagen) and 5 nmol of DsiRNA NLRP3, with the following sense sequence: 5'-AAC CUG CUU CUC ACA UGU CGU CUG UAC-3'. The knock-down efficiency was confirmed using FCS with specific antibody for NALP3 and the specificity was proved using a control - negative siRNA (Qiagen).

Real-time PCR analyses: Total RNA was isolated from primary or 15P-1 Sertoli cells using RNeasy mini kit (Qiagen). First strand cDNA was synthesized using Sensiscript Reverse Transcriptase (Qiagen), following the manufacturer's protocol. Quantitative amplification was performed on a MX3500P cycler real-time RT-PCR system (Stratagene) using EvaGreen Mastermix (Geneaxxone). The melting curve of each PCR product was checked for specific amplification. Data were normalised for the expression of β-Actin in each experiment. Data are presented as relative expression following the ΔΔCt method. The primers were obtained using primer design software with thermodynamic optimisation and miss-priming and self-dimmer evaluation (Primer Design): occludin: 5'-ACTATGCGGAAAGAGTTGACAG-3' and 5'-GTCATCCACACTCAAGGTCAG-3'; actb: 5'-CTGGGAGTGGGGAGGC-3' and 5'-TCAACTGGTCTCAGTG-3'

Flow cytometry: Control and treated Sertoli cells were detached with 0.02% EDTA and washed with cold PBS plus 1% BSA. For detection of NALP3 (knockdown experiments) and occludin, the specific antibodies were used (anti-rabbit cryopyrin, clone H-66, anti-goat occlu-

din, clone Y-12, Santa Cruz). After 4% PFA fixation/permeabilization solution wash, and staining solution block (eBiosciences), the specific primary Abs or the appropriate isotypic control Abs were used at concentration of 0.5 μg/10^6 cells for 30 min on ice, followed by BSA-PBS wash, second antibody incubation at concentration of 0.25 μg/10^6 cells for 30 min on ice (in dark). The cells were analyzed with a FACSCalibur flow cytometer (Becton Dickenson). Fluorescence of 10^4 cells per sample was acquired in logarithmic mode for visual inspection of the distributions and for quantifying the expression of the relevant molecules by calculating the fluorescence intensity quadratic mean.

Immunofluorescence: Both primary and 15P-1 Sertoli cells were grown on glass coverslips and treated for 24h with ie-DAP and MDP. Control cells were non-treated with both ligands. After incubation, cells were washed with PBS, fixed with 4% paraformaldehyde for 10-15 min and subsequently permeabilized with 0.2% Triton X-100 in PBS for 15 min at RT. After blocking (1% BSA-PBS) incubation at RT for 1h, the Sertoli covered coverslips were probed with anti-goat occludin antibody diluted 1:50 and incubated overnight, at 4°C. Negative control cells were non-incubated with primary antibody. After 3 wash steps with PBS, secondary mouse anti-goat IgG-FITC (sc-53800) were used at 1:50 dilution, for 1h, RT (dark), and extensively PBS washed and Vectorshield-PI mounting media included. Slides were visualised with a confocal laser-scanning microscope (Leica Microsystems).

Statistical Analysis: The data was generated from three independent experiments, each performed in triplicates. One-way or Two-way ANOVA tests with respective multiple comparison post-tests were used (GraphPad Prism 6 software). $P < 0.01$ was considered significant.

RESULTS

In primary prepubertal Sertoli cells (15 days), the ie-DAP treatment induced significant up regulation of occludin mRNA transcripts compared to non-treated cells. MDP treatment also resulted in significant increase in occludin gene expression but compared to specific NOD1 ligand challenged it was decreased. Combination of NOD1 and NOD2 ligand challenge resulted in significant increase in occludin mRNA expression in primary Sertoli cells (Fig. 1A). Protein expression of occludin followed similar pattern. After ie-DAP treatment the occludin levels were significantly up-regulated in the prepubertal cells compared to non-treated cells (Fig. 1B). Occludin mRNA expression was significantly increased after ie-DAP treatment in adult Sertoli cells (15p1 cell line). There was significant increase in mRNA levels after MDP and combination of both compounds challenge compared to non-treated cells but significantly lower compared to ie-DAP treatment alone (Fig. 1C). We observed interesting phenomenon in occludin protein expression levels with two different cell populations after ieDAP and MDP treatment (Fig. 1D). The silencing of NALP3 in adult Sertoli cell line 15P-1(Fig. 2B) resulted in up-regulation of occludin transcript levels compared not only to negative control cells but also to NALP3 knockdown and NOD1/2 ligand challenged cells (Fig. 2A). We detected increase occludin protein levels after NALP3 silencing and in case of knockdown and combination of ie-DAP and MDP treatment (Fig. 2C). We observed positive occludin expression in ie-DAP and MDP treated primary and 15P-1 Sertoli cells. There was no occludin expression in control non-treated cells (Fig. 3).

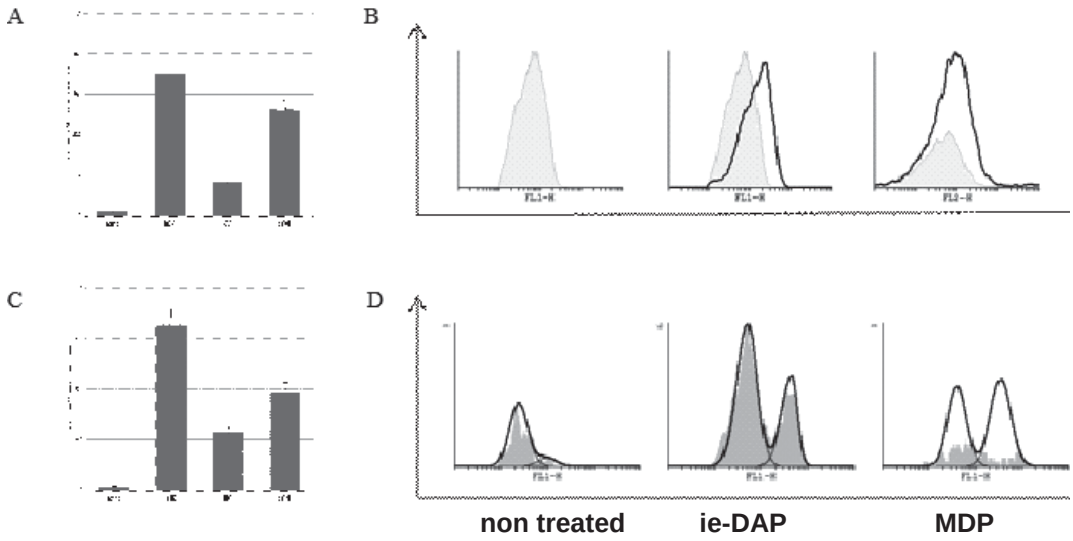

Fig. 1. Sertoli cells were treated with ie-DAP and MDP, and combinations of both compounds. Quantitative PCR assessment of mRNA transcripts encoding mouse occludin mRNA in (A) primary cells (C) adult Sertoli cells. Y-axis represents the relative mRNA expression fold change, assessed using the ΔΔCt method, and normalized by the expression of the constitutive housekeeping gene Actin-β. Flow cytometry assessment of protein abundance of occludin after challenge with iE-DAP and MDP in (B) primary cells and (D) adult Sertoli cells. Expression is evaluated by the MFI indexes from the histogram overlay analysis. Data are expressed as MFI fold induction. P < 0.01

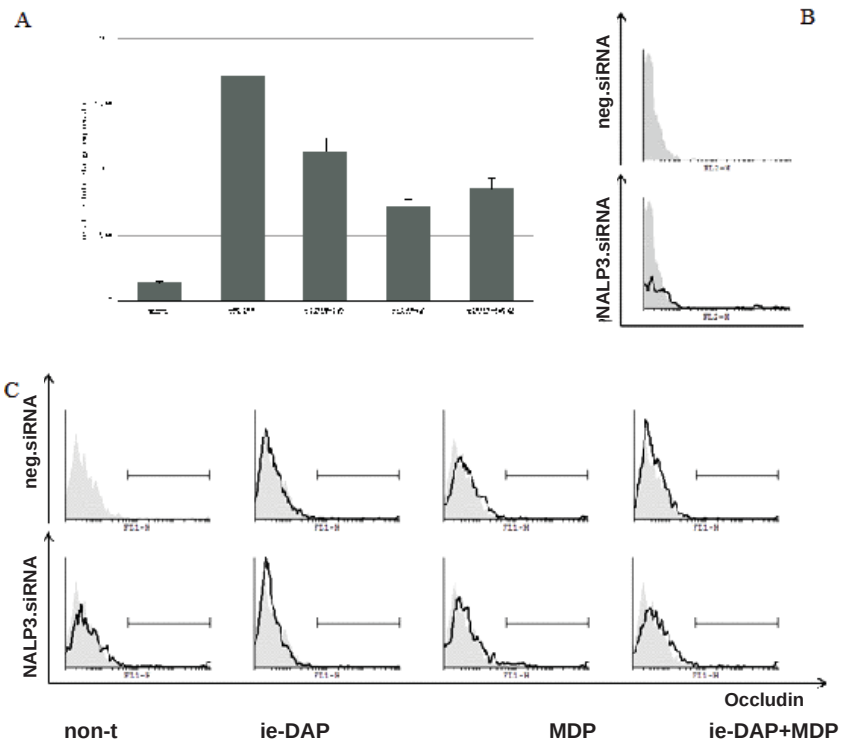

Fig. 2. Sertoli cell line 15-P1 was silenced with NALP3 siRNA and treated with ieDAP, MDP, and combination of both compounds. (A) Quantitative PCR assessment of mRNA transcripts encoding mouse occludin mRNA. Y-axis represents the relative mRNA expression fold change, assessed using the ΔΔCt method, and normalized by the expression of the constitutive housekeeping gene Actin-β. (B) The silencing of NALP3 in adult Sertoli cell line 15P-1 using transfection with validated hi-efficiency DsiRNA against its exon 5 CDS; (C) Flow cytometry assessment of protein abundance of occludin. Expression is evaluated by the MFI indexes from the histogram overlay analysis. Data are expressed as MFI fold induction. P < 0.01

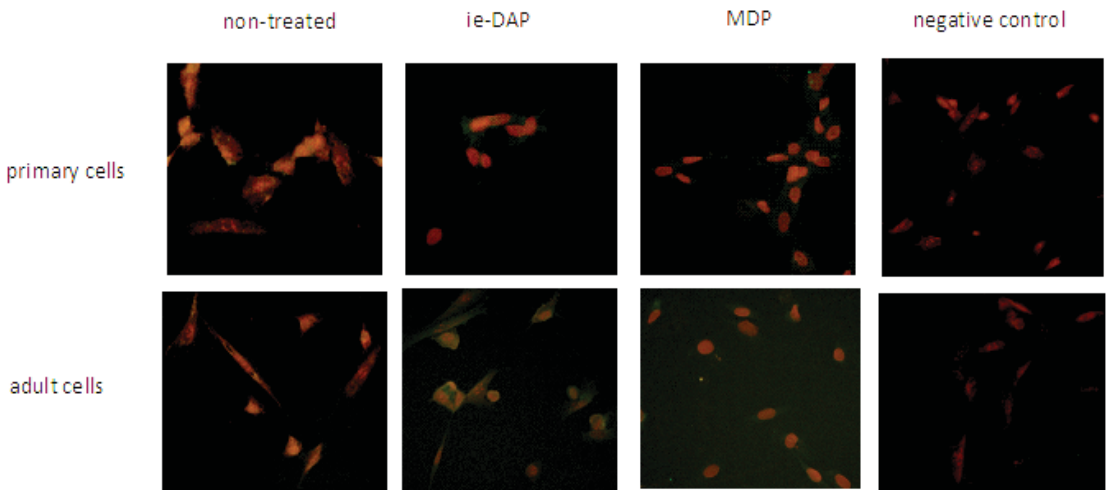

Fig. 3. Immunofluorescent detection of occludin expression in non-treated and ie-DAP and MDP treated primary and adult (15-P1) Sertoli cells, using goat anti-human occludin polyclonal antibody and donkey anti-goat FITC conjugated secondary antibody. Negative controls of irrelevant goat IgG treated specimens, probed and the same secondary antibody are also present. ie-DAP and MDP treatment results in an increased occludin green expression localized in the cell cytoplasm

DISCUSSION

Sertoli cells nourish the developing sperm cells and act as phagocytes, consuming the residual cytoplasm during spermatogenesis. Infections of the testis are relatively rare in comparison with more distal tissues of the male reproductive tract [7]. This may be due to an increased reliance on innate immunity, and there has been a steady increase in interest in the role of innate immunity in testicular function recently [2, 4, 8]. The system of NOD1, NOD2 receptors and their downstream targets – NALP3 are part of the inflammasome and have been implicated so far in many autoimmune disease in men. Claudins [5] and occludin [6] were the first tight-junction integral membrane proteins identified. In this study, the blood-testis barrier crucially important tight-junction protein occludin spanning in-between the cell-cell junction interface was found upregulated under challeging either NOD1 or NOD2 receptors, or both. Inflammasome NALP3 silencing had restrictive effect on this upregulation and it is connected with occluding expression levels. The innate immunity and tight-junction pathways integration eventually serve a protective role for both testis immune barrier and spermatogenesis compartmentalisation maintained by the very same barrier. This integration also points the way for mechanistic research of the disturbances inflicted during an inflammatory response in testis niche.

Acknowledgments:

This study was partially funded by internal grant attributed to Dr. Soren Hayrabedyan (Project “Sertoli cell innate immunity”) under 7FP ReProForce.

Conflict of Interests:

The authors declare that there is no conflict of interests regarding the publication of this paper.

REFERENCES

1. Benetti, E., F. Chiazza, et al. The NLRP3 Inflammasome as a Novel Player of the Intercellular Crosstalk in Metabolic Disorders. – Mediators of Inflammation. 2013, Article ID 678627, 9 pages, 2013. doi:10.1155/2013/678627.
2. Bhushan, S., S. Tchatalbachev et al. Uropathogenic Escherichia coli block MyD88-dependent and activate MyD88-independent signaling pathways in rat testicular cells. – J. Immunol., 180, 2008, 5537-5547.
3. Chung, N. P. Y., C. Y. Cheng. Is cadmium chloride-induced inter-Sertoli tight junction permeability barrier disruption a suitable in vitro model to study the events of junction disassembly during spermatogenesis in the rat testis? – Endocrinology, 142, 2001, 1878-1888.
4. Com, E., F. Bourgeon et al. Expression of antimicrobial defensins in the male reproductive tract of rats, mice, and humans. – Biol. Reprod., 68, 2003, 95-104.
5. Furuse, M., K. Fujita et al. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. – J. Cell Biol. 141, 1998, 1539-1550.
6. Furuse, M., T. Hirase et al. Occludin: a novel integral membrane protein localizing at tight junctions. – J. Cell Biol. 123, 1993, 1777-1788.
7. Krieger, J. N. Epididymitis, orchitis, and related conditions. – Sex. Transm. Dis., 11, 1984:173-181.
8. Starace, D, R. Galli et al. Toll-like receptor 3 activation induces antiviral immune responses in mouse Sertoli cells. – Biol. Reprod., 79, 2008, 766-775.
9. Turner, C. M., N. Arulkumaran, et al. Is the inflammasome a potential therapeutic target in renal disease? – BMC Nephrology, 2014, 15-21.

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