

**THE EFFECT OF CD300A RECEPTOR ON CASPASE-1
ACTIVITY IN THE CONTEXT OF CELL DEATH
AND ON ITS ACTIVATORS Nlrp3 AND Asc
IN SERTOLI CELLS**

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Abstract

The phagocytosis of spermatozoa cytoplasm by Sertoli cells is a key maturation event during spermiation and takes place in a blood-testis barrier maintained immune-privileged environment that is essential for the prevention of the sperm self-antigens from presentation. We have recently discovered Nlrp3 inflammasome to be functional in Sertoli cells upon TLR4 and NOD receptors challenge in ATP danger presence, resulting in release of pro-inflammatory cytokines and cell death that could potentially result in breaking of the self-tolerance. In this context we have investigated, the considered inhibitory, phagocytic receptor CD300a that is able to detect eventually sperm cell surface apoptotic signals. We found CD300a to be essential for Nlrp3 inflammasome activation via CD300a-dependent Asc expression and both caspase-1 and caspase-3 activation, since its gene silencing abrogated these events. At the same time CD300a demonstrated bi-directional regulatory abilities, suppressing Gasdermin D inflammasome effector pro- and activated forms. CD300a had a restrictive effect on cell population level reducing caspase positive cells in strong caspase-1 and 3, and weak caspase-3 expressing dead cells, suggesting key regulatory role in Sertoli cell faith. CD300a should be further explored as

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its potential perturbation could play a role in putative self-antigen-tolerance perturbation and thus contribute to male infertility.

Key words: Sertoli cells, caspase, inflammasome, cell death

Introduction. Most frequently, clinical male infertility is associated with asymptotic idiopathic spermatogenesis perturbation that resembles closely the pathological findings in animal experimental autoimmune orchitis models caused by live syngeneic spermatogenic cells auto-immunization [1,2]. Sertoli cells nourish the germline in seminiferous tubules, sustaining the blood-testis barrier (BTB) – a physical and paracrine immune privileged tolerogenic environment, protecting spermatogonia self-antigens from antigen-presentation. In some cases, these epithelial cells could also serve as a non-professional antigen-presenting cells, and as we have recently established, they could elicit a Nlrp3 inflammasome response, secreting the pro-inflammatory cytokines IL-1 β and IL-6, as well as undergoing caspase-1 dependent cell death [3]. *Spermiation* takes major part in sperm cell maturation, consisting of constant phagocytosis of most of the spermatozoa cytoplasm by the Sertoli cells. This makes Sertoli phagocytic function another crucial step in preserving sperm antigen self-tolerance. Specialized receptors, like the ones in the CD300 family of type I transmembrane glycoproteins, distinguish live from pro-apoptotic cells by recognizing phosphatidyl serine flip-flop phenomena on cell membrane surface. Our recent data (unpublished RNA-seq studies) have revealed a significant CD300a upregulation upon a NOD family innate immune receptor challenge, known to converge downstream in a subsequent NF- κ B activation [3]. CD300a is expressed on many phagocytosing immune cells, and the LPS challenge of the similarly converging to NF- κ B downstream innate immune receptor TLR4, has been found to result in huge CD300a activation [4], despite later being widely considered an inhibitory molecule in myeloid and other cell types [5,6, and crucial for TLR4 induced IL-6 secretion [7]. Since TLR4 induction in presence of the danger signal ATP resulted in Nlrp3 activation, caspase-1 cleavage and pro-inflammatory cytokine production, it is very important to follow the role of CD300a in a putative Nlrp3 inflammasome dependent subsequent cell death caspase and Gasdermin D activation. The *aim* of this study was to investigate if CD300a expression has an impact on TLR4 mediated ATP danger signal inflammasome and apoptotic cell death that could potentially change the tolerogenic environment balance favouring self-antigen presentation.

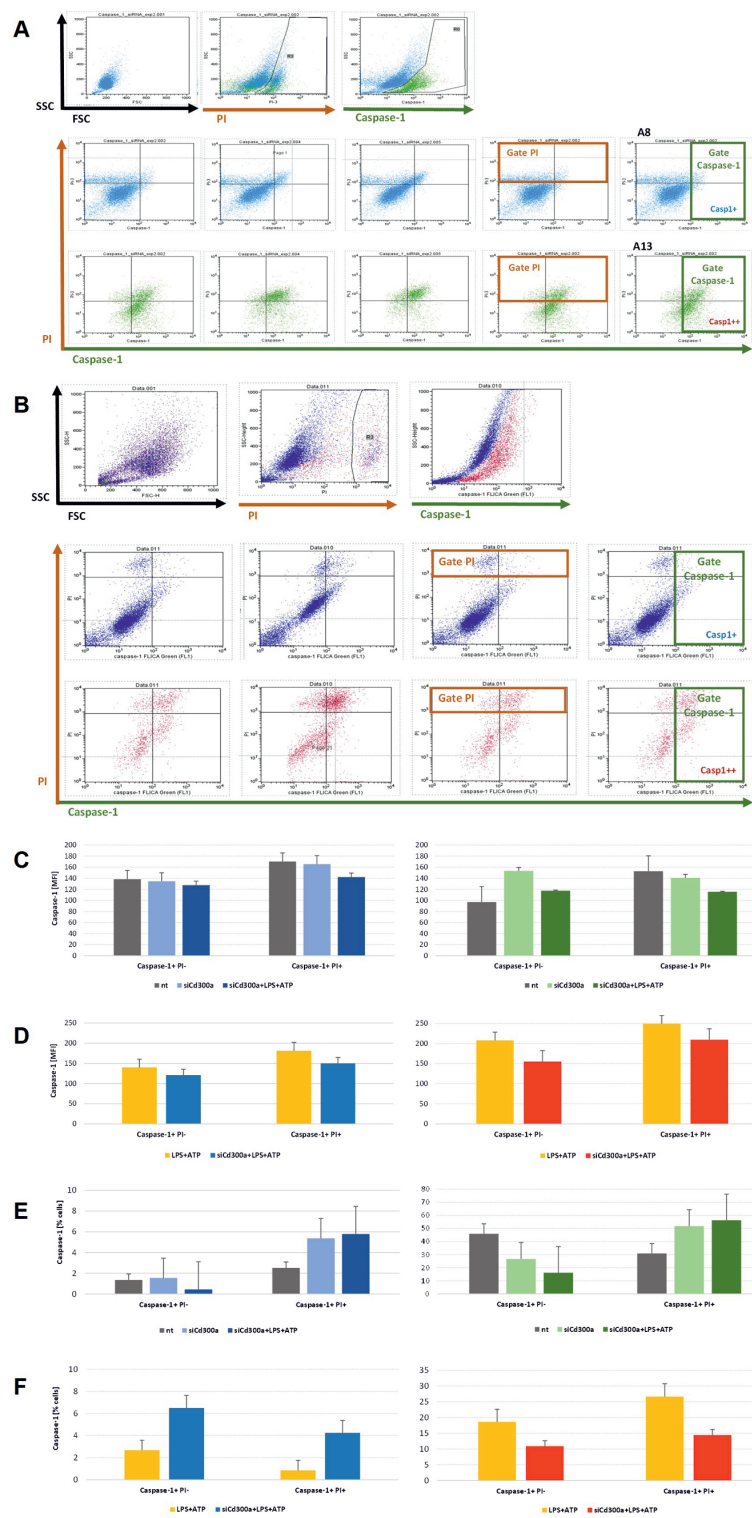
Materials and methods. Mouse cell line 15P-1 of Sertoli cells (ATCC) was cultured in 12/24 well plates in complete growth medium DMEM, high glucose (4.5 g/L), 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, 10% FBS and 1% Pen-Strep sol. Gene silencing of CD300a gene was carried out for 48 h, using transfection of 10 μ M enhanced small interfering RNA (DsiRNA, IDT Inc.). Some cells were additionally challenged for 24 h with LPS (2 mg/mL) and for 2 h with ATP (5 μ M). To detect caspase-1 and caspase-3 activity, cells were then

treated with FAM FLICA Caspase-1 Assay Kit, or FAM FLICA 660 Caspase-3 Assay Kit (ImmunoChemistry Tech., US), accordingly, and co-stained with dead cells dye PI (250 µg/mL). Cells were then analyzed using flow cytometry following standard protocol described elsewhere [3]. Total RNA was isolated from similarly CD300a silenced cells, using Quick-RNA Miniprep Kit (ZymoResearch), converted to cDNA using an iScript™ Select cDNA Synthesis Kit (Bio-Rad), and used as a template in comparative RT-qPCR reaction to detect Nlrp3 and Asc gene expression, using an iTaq™ Universal SYBR® Green Supermix (Bio-Rad) and specific primers (IDT DNA Technologies). The protein expression of the total Gasdermin D and its C-terminal cleaved form released upon activation in Sertoli cell lysates were assessed using GSDMD ELISA Kit (Abcam) and GSDMD-CT ELISA Kit (Adipogen), respectively.

Results. The adult Sertoli 15P-1 cell line was used to model the impact of the CD300a on the caspase-1 and caspase-3 activity using its functional gene silencing via siRNA in the context of TLR4 and inflammasome challenge, using LPS and ATP accordingly (Fig. 1, 2). Activated caspase-1 and caspase-3 positive cells were emitting green and red fluorescence, respectively, as intense as the number of activated caspase molecules per cell, at the moment of addition of caspase-specific FLICA substrates used. Two intensity-distinct, caspase-1-positive populations were identified and analyzed separately. We have referred them to as a “caspase-1 weakly activated” (Casp1+) and “caspase-1 strongly activated” (Casp1++) (Fig. 1A, B). Since caspase-1 and caspase-3 induction often results in cell death, we consider live and dead cell populations separately.

CD300a inhibitory effect is reduced or lost in caspase-1 activated, dead cells. Sertoli cells that had weakly activated caspase-1 (Casp1+) were less affected by the CD300a silencing as there was no significant difference between the intact and siCD300a treated cells in both caspase-1 live and dead populations (Fig. 1C). In contrast, CD300a silencing in caspase-1 strongly activated popula-

Fig. 1. Representative experiments of Flow cytometry scatter charts of investigated samples for caspase-1 activity and population distribution. A. 1st row: Forward vs Side Scatter (SSC), Propidium Iodide (PI, FL2) vs SSC, Gate: caspase-1 FLICA (FL1) vs SSH – two Casp populations defined. 2nd and 3rd row – definition of Casp1+ and Casp1++ populations, by weak/strong caspase-1 intensity: non-treated, siCD300a silenced, siCD300a silenced and treated with LPS and ATP, dead cells gate for population 1/2, caspase-1 gate for Casp1+/Casp1++ populations. B. 1st row: FSC vs SSC, (PI, FL2) vs SSC, Gate: caspase-1 FLICA (FL1) vs SSC – two Casp1 populations defined. 2nd and 3rd row – definition of Casp1+ and Casp1++ populations, by weak/strong caspase-1 intensity: LPS and ATP treated, siCD300a silenced and treated with LPS and ATP, dead cells gate for Casp1+/Casp1++ population, caspase-1 gate for Casp1+/Casp1++ population. C., D. Bar charts of caspase-1 intensity assessed as Median Fluorescent Index (MFI) in caspase-1+ PI- and caspase-1+ PI+ respective regions. E., F. Bar charts of caspase-1 positive populations in caspase-1+ PI- and caspase-1+ PI+ respective regions. Data is presented for two subpopulations Casp1+ and Casp1++ on the left and on the right accordingly →



tion (Casp1++) significantly increased caspase-1 activity in live cells, while there was no significant effect in dead cells. When both Casp1+/Casp1++ populations were CD300a silenced prior to TLR4 challenged, there was again difference in their response (Fig. 1D).

The Casp1+ live population had no significant change in caspase-1 activity, while Casp1++ live population demonstrated significant increase in the caspase-1 activity compared to intact live cells, but this activity was still lower than the one induced in the intact CD300a silenced cells. Both CD300a silenced, caspase-1 positive dead populations (Casp1+ and Casp1++) exhibited a significant decrease in the caspase-1 activity post TLR4 challenge. Based on this data, CD300a could be considered restrictive to caspase-1 activity in Casp1++ live population and pro-active in TLR4 challenged dead cells in both Casp1+/Casp1++ populations (Fig. 1C). Interestingly, when cell distribution of the live and dead populations, positive for caspase-1 was considered (based on percentage of positive events), we found CD300a silencing to result in significant caspase-1 activity increase in both Casp1+ and Casp1++ dead populations, as well as in Casp1+ live population, while TLR4 challenge addition had no further effect in both dead populations (Fig. 1E). In Casp1+ population, silencing of CD300a reduced caspase-1 significantly in intact cells, and even more in TLR4 challenged cells. This TLR4 induced caspase-1 activity abrogation in CD300a silenced context was in fact observed in the Casp1+ live population as well. This data suggests that in the absence of CD300a more non-challenged, dead cells are activated, while live cells, both non-challenged and challenged alike, are less activated (Fig. 1E).

We then focused on the effect of CD300a silencing on TLR challenge (comparing TLR challenge in intact and CD300a silenced cells) and we found that in terms of caspase-1 intensity of activation it was abrogated in both Casp1++/Casp1+ populations, in live and dead cells alike, as if CD300a would have a proactive effect on this caspase activity (Fig. 1D). In comparison, the caspase-1 intensity was higher in both Casp1+/Casp1++ dead cell populations, compared to their live counterparts, as well as it was higher in Casp1++ vs Casp1+ dead populations (Fig. 1C). When cell populations were considered percentage of affected cells, the Casp1+ population was found different to Casp1++ population (Fig. 1F). CD300a silencing resulted in an increased number of caspase-1 positive cells in Casp+ population, regardless the cells were dead or alive, while in Casp1++ population, the same silencing resulted in a decrease of caspase-1 positive cells. Overall, despite some ambiguity, CD300a silencing in TLR4 challenged cells resulted in a significantly decreased number of caspase-1 positive cells, with a decrease in caspase-1 intensity as well, both in live and dead cells alike, as if it is caspase-1 pro-activator. The only exception was Casp+ population of live cell, where CD300a behaved as an inhibitor.

CD300a inhibitory effect is lost regarding caspase-3 activation, and only preserved in weak caspase-3 positive dead population. We have

investigated caspase-3 activation in CD300a silenced context using similar approach, revealing a prominent two Casp3+ and Casp3++ populations, based on weak and strong caspase-3 activation (assessed by caspase-3 intensity using histogram MFI), especially when challenging TLR4 in CD300a silenced conditions (Fig. 2A). We found that in both live and dead, weakly caspase-3 activated population (Casp3+), as well as in the live, strongly caspase-3 activated population (Casp3++), there was no significant change in the caspase-3 intensity upon TLR4 challenge in CD300a silenced context, when compared to TLR4 challenge alone (Fig. 2B).

In Casp3++ dead cells caspase-3 activation upon TLR4 challenge was higher compared to live caspase-3 positive population, as well as compared to Casp3+ dead population. This caspase-3 activation, measured as MFI of histogram intensity, was in fact decreased when CD300a was silenced before the TLR4 challenge was done (Fig. 2B). In regard to caspase-3 positive cell population distribution, both Casp3+ and Casp3++ live populations decreased in caspase-3 positive cells, when TLR4 was challenged in CD300a silenced conditions, compared to TLR4 challenged alone, suggesting CD300a involvement in TLR4 induction of the caspase-3 activation. Rather opposite phenomena were observed in the dead caspase-3 populations in both Casp+ and Casp++ cells. TLR4 challenge in CD300a silenced 15P-1 Sertoli cells resulted in higher percentage of caspase-3 positive cells when compared to TLR4 challenge alone, especially in Casp+ population of dead cells (Fig. 2B). Overall, CD300a silencing in caspase-3 dead cells resulted in a marked increase in the number of caspase-3 positive cells, within the weak intensity population (Casp3+), but at the same time it resulted in a reduced caspase-3 intensity in the strong intensity population (Casp3++).

CD300a activity is directly linked to inflammasome adapter and Gasdermin D expression. We further investigated if major inflammasome components such as inflammasome scaffold coding gene *Nlrp3* and inflammasome adaptor coding gene *Asc* were linked to CD300a expression. Silencing CD300a, we found that *Nlrp3* transcripts were not affected, but the *Asc* transcript levels declined more than 60% (Fig. 3A, B), suggesting direct link between the CD300a levels of expression and *Asc* levels of expression. We used two sandwich ELISA assays to assess quantitatively the levels of expression of total Gasdermin D (Fig. 3C) and its C-terminal cleaved form (Fig. 3D). Upon caspase-1 activation, as main classical Gasdermin activation pathway, Gasdermin D pro-form is cleaved into N-terminal form that comprises Gasdermin-D based multimer pores penetrating plasma membrane and leading to pyroptotic cell death, and C-terminal form released via those pores out of the cell. We found that CD300a silencing prevented Gasdermin D pro-form upregulation in intact and TLR4 challenged conditions (Fig. 3C), while Gasdermin D cleaved form was even further reduced compared to basal cleavage levels (Fig. 3D), suggesting that CD300a is required to sustain Gasdermin D pro-form levels, and eventually for its cleavage and activation.

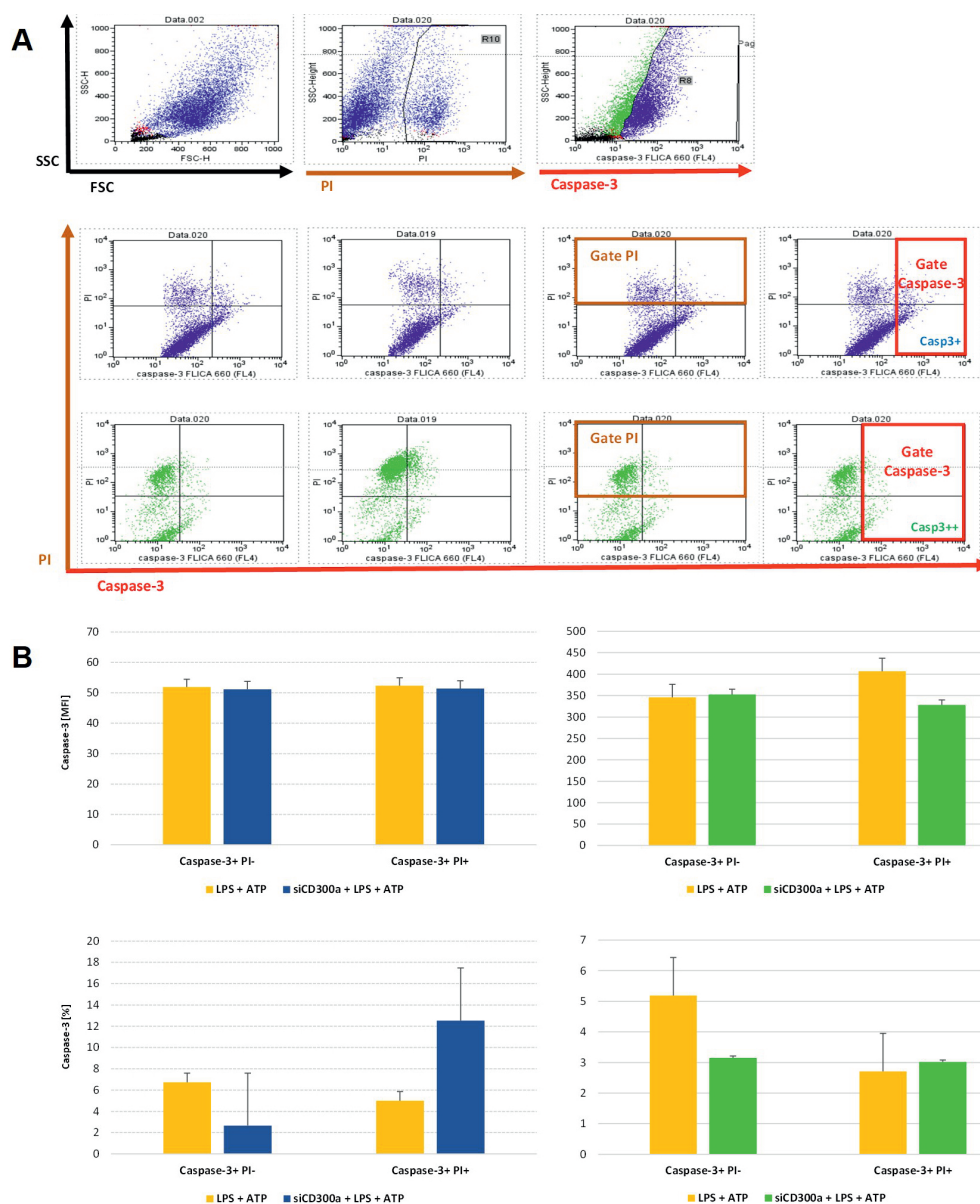


Fig. 2. Representative experiments of Flow cytometry scatter charts of investigated samples for caspase-3 activity and population distribution. A. 1st row: FSC vs SSC, PI (FL2) vs SSC, Gate: caspase-3 FLICA 660 (FL4) vs SSH – two Casp3 populations defined. 2nd and 3rd row – definition of Casp3+ and Casp3++ populations, by weak/strong caspase-3 intensity: LPS and ATP treated, siCD300a silenced and treated with LPS and ATP, dead cells gate for Casp3+/Casp3++ populations, caspase-3 gate for population Casp3+/Casp3++ Casp3+/Casp3++. B. Bar charts of caspase-3 intensity assessed as MFI in caspase-3+ PI- and caspase-3+ PI+ respective regions. D. Bar charts of caspase-1 positive populations in caspase-3+ PI- and caspase-3+ PI+ respective regions. Data is presented for two subpopulations Casp3+ and Casp3++ on the left and on the right accordingly

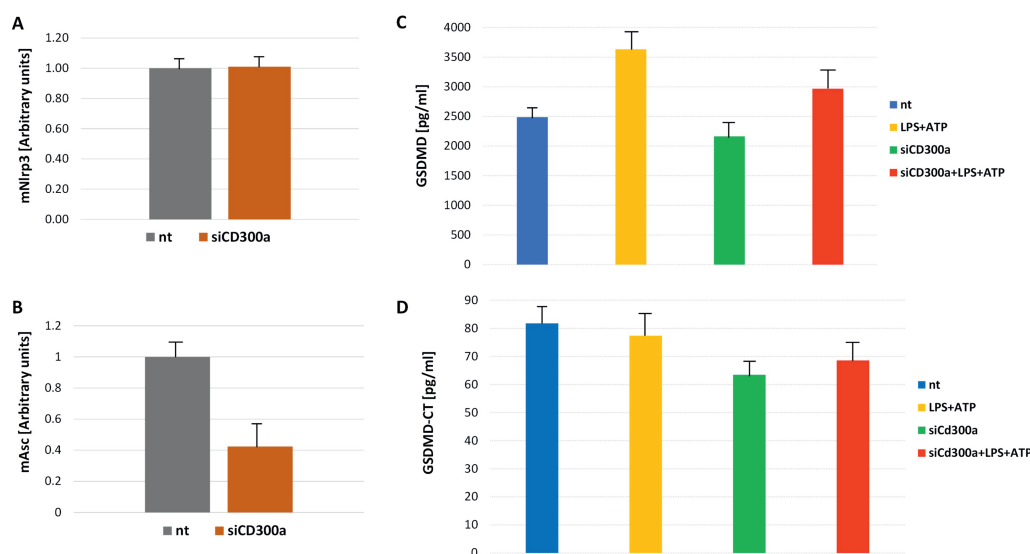


Fig. 3. A., B. Effect of siRNA CD300a silencing on Nlrp3 and Asc mRNA expression, respectively, assessed using comparative RT-qPCR. mRNA expression is represented as normalized relative expression to non-treated cells. C., D. Sandwich ELISA for assessing the total Gasdermin D and cleaved Gasdermin D C-terminal subunit protein abundance, respectively, in non-treated, LPS and ATP treated, CD300a siRNA silenced and in CD300a silenced and LPS and ATP treated 15P-1 cells

Discussion. Constant phagocytosis of spermatozoa cytoplasm by the Sertoli cells holds potential for self-antigen presentation upon disruption in the local immune tolerance. In this context, a major role is played by molecules that regulate the cellular innate immune signalling/response, in an event of phagocytic activity, thus preventing an excessive pro-inflammatory cytokine release that could compromise the BTB. We have recently shown, for the first time in Sertoli cells, a functional Nlrp3 inflammasome able to elicit pro-inflammatory cytokine response and cell death, upon stimulation of the NF- κ B converging NOD1 and TLR4, in the presence of danger signal as ATP that could serve as a cornerstone for BTB disruption and potential infertility upon proper pathogenic stimulation [8]. We have also found that NOD1 challenge strongly upregulated the CD300a receptor (*unpublished*), which recognizes phosphatidylserine and phosphatidylethanolamine presented on apoptotic cell membranes [8]. CD300a is a member of the CD300 type I trans-membrane glycoproteins, signalling via its ITIM motif and being regulated by internalization [9,10]. CD300a is expressed on both mice and human myeloid cells, NK, T cell subsets, Ne, Eo, Mo, dendritic cells, and Ba cells [6], and so far, it has been considered an inhibitory receptor in myeloid derived cells, although new studies revealed that TLR4 challenge in Ne strongly upregulates CD300a, and its signalling is also NF- κ B dependent [4]. In this study, we challenged the TLR4 receptor pathway, also downstream mediated by NF- κ B, and we found that

pro-caspase-1 activation was CD300a-dependent, demonstrated by the caspase-1 FLICA probe intensity decline in CD300a-silenced conditions. Similarly, caspase-3 followed the same pattern. We further found that CD300a is required for inflammasome adaptor protein Asc expression, thus being crucial for the Nlrp3 and other inflammasomes assembly, since all but one use Asc to activate caspase-1. In this context we found that TLR4 challenge of Sertoli cells separated them into very strong and moderate caspase-1 and caspase-3 distinct responder populations.

We found also another regulation pattern for CD300a – sometimes it behaved as negative regulator for caspase-1/-3 activation, limiting the rate of activation in intact cell population exhibiting a high basal caspase-1 expression already, but also on population level, reducing the size of dead populations, positive for caspase-1 and caspase-3. We found that CD300a behaved as bi-directional regulator in TLR4 challenged Sertoli cells – in strong responder population, with many active caspase-1 molecules per cell, CD300a served as an inflammasome assembly enhancer, while in the weakly responding cells, with lesser activated caspases, it served as an inflammasome restrictor. Dead, caspase-3 strongly activated cells, matched caspase-1 pattern, suggesting that CD300a enhancing function can potentially pass the signal from caspase-1 towards more distant cell death pathways such as apoptotic one. CD300a is proptosis restrictive, affecting negatively both total and cleaved Gasdermin-D, meaning that CD300a might promote caspase-1 pathway, but again bi-directionally restrict massive cell death. Similarly, CD300a is considered negative regulator in other phagocytic cell types, as CD300a gene deletion in Ma results in a prolonged proinflammatory IL-6 secretion [7], but on the other hand, TLR4 activation in Ne, results in CD300a upregulation and strong ROS expression [4], as well as CD300a has been found crucial to fight off an UPEC in urogenital tract [8]. Thus, our data suggest that phagocytic receptor CD300a signalling perturbation could potentially impact Sertoli cell faith and antigen presentation via caspase-1 and caspase-3 activity modulation.

Conclusion. We found CD300a to be a novel Nlrp3-caspase-cell death bi-directional regulator that can serve potentially as defender but also as death enhancer molecule, thus making further research of its mechanistic action and caspase-1 and caspase-3 interaction imminent and crucially important for Sertoli blood-testis barrier maintenance.

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