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Modulation of γδ T cell activation by neutrophil elastase

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Key words: γδ T cells, neutrophils, neutrophil serine proteases, elastase, proteases-activated receptor.

Abbreviations:

HMBPP: (E)-1-hydroxy-2-methylbut-2-enyl 4-diphosphate

PAR: proteases-activated receptor

ROS: reactive oxygen species

Abstract

γδ T cells are non-conventional, innate-like T cells, characterized by a restricted TCR repertoire. They participate in protective immunity response against extracellular and intracellular pathogens, tumor surveillance, modulation of innate and adaptive immune responses, tissue healing, epithelial cell maintenance, and regulation of physiological organ function. In this study, we investigated the role of neutrophils during the activation of human blood γδ T cells through CD3 molecules. We found that the up-regulation of CD69 expression, and the production of IFN-γ and TNF-α induced by anti-CD3 antibodies were potentiated by neutrophils. We found that inhibition of caspase-1 and neutralization of IL-18 did not affect neutrophil-mediated modulation. By contrast, the treatment with serine proteases inhibitors prevented the potentiation of γδ T cell activation induced by neutrophils. Moreover, the addition of elastase to γδ T cell culture increased their stimulation, and the treatment of neutrophils with elastase inhibitor prevented the effect of neutrophils on γδ T cell activation. Furthermore, we demonstrated that the effect of elastase on γδ T cells was mediated through the

proteases-activated receptor, PAR1, since the inhibition of this receptor with a specific antagonist, RWJ56110, abrogated the effect of neutrophils on γδ T cell activation.

Introduction

γδ T cells are non-conventional, innate-like T cells, characterized by a restricted TCR repertoire. γδ T cells recognize self and non-self molecules in a non-MHC restricted manner.[1] They exert a variety of functions, that include protective immunity against extracellular and intracellular pathogens, tumor surveillance, modulation of innate and adaptive immune responses, tissue healing and epithelial cell maintenance, and regulation of physiological organ function.[2,3] Vγ9Vδ2 T cells represent the major γδ T cell subset in human peripheral blood where they comprise 1-10% in healthy adults. Vγ9Vδ2 T cells acquire a pre-activated phenotype early in their development allowing the rapid induction of a wide variety of functions following the detection of activating signals.[4,5] Among them, they can exert a high cytotoxic response against infected and transformed cells, produce cytokines and chemokines, regulate the recruitment and the activation of neutrophils, induce Th1 polarization, promote the activation of B cells, and the presentation of antigenic peptides to T cells.[6-9]

Neutrophils are the most abundant leukocytes in human peripheral blood.[10] They are innate immune cells that play a key role in immunity against extracellular pathogens.[11] At infected tissues, neutrophils represent the first immune cells recruited by the local production of chemokines such as CXCL8. Neutrophils act as phagocytic cells, and they can destroy microbes through the action of oxidative and non-oxidative pathways such as lytic enzymes, antimicrobial peptides and by the production of reactive oxygen

species (ROS).[11-13] In the non-oxidative pathway of intracellular and extracellular pathogen destruction participates the serine proteases: elastase, proteinase 3 and cathepsin G. In addition to the role of serine proteases in pathogen destruction, they are involved in the regulation of proinflammatory responses and in a variety of chronic inflammatory diseases, such as chronic obstructive pulmonary disease, cystic fibrosis, acute lung injury, and acute respiratory distress syndrome.[14-16] At inflammatory sites, neutrophil serine proteases are secreted into the extracellular milieu in response to inflammatory signals such as TNF-α, CXCL8 and LPS, among others.[17] A fraction of the released proteases remain bound in an active form on the external surface of the plasma membrane. The soluble and membrane bound proteases can proteolytically regulate the activities of a different chemokines, cytokines, growth factors, and cell surface receptors.[18,19] Neutrophils serine proteases can cleave the N-terminal extracellular domain of proteases-activated receptors (PARs), revealing a tethered ligand that allows the autoactivation of the receptor.[20] PARs are ubiquitously expressed in various tissues and cells,[21-24] and they are a subfamily of related G-protein-coupled receptors.

It has been reported that neutrophils can modulate the activation of conventional T cells (TCRαβ),[25-28] and we recently demonstrated that neutrophils also regulate the activation of γδ T cells induced by the phosphontigen HMBPP ((E)-1-hydroxy-2-methylbut-2-enyl 4-diphosphate).[29] In this study, we analyzed whether neutrophils were able to modulate the phenotype and function of human blood γδ T cells activated through CD3 molecules. Our data demonstrate that, under these conditions, neutrophils potentiate the activation of γδ T cells, and this effect was mediated through the action of neutrophil elastase on the proteases-activated receptor, PAR1.

Materials and methods

Reagents and antibodies

Ficoll-Hypaque and dextran was from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Anti-TCR γ/δ MicroBead kit was obtained from Miltenyi Biotec (Germany). RPMI 1640 medium, fetal bovine serum (FBS) and DHR (dihydrorhodamine 123) were from Invitrogen (Carlsbad, CA, USA). Anti-CD3 monoclonal antibodies (UTCH-1) were obtained from Beckman Coulter (Marseille, France). Phycoerithrin (PE)-conjugated mouse anti-CD11b, PE.Cy5-conjugated mouse anti-CD69, and isotype controls were from BD Bioscience (San Jose, CA). PE-conjugated anti-PAR1 monoclonal antibodies (ATAP2) and PAR1 antagonist RWJ56110 were from Santa Cruz Biotechnology (Dallas, TX). Rabbit anti-human elastase antibody was from Calbiochem (Massachusetts, MA), DyLight 549-conjugated goat anti-rabbit IgG and isotype matched antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Mouse anti-human CD107a-PE was from BioLegend (San Diego, CA). Human IFN-γ and TNF-α ELISA kit was from BD Bioscience (San Diego, CA). Human interleukin (IL)-18 detection antibodies, and blocking IL-18 antibodies (clone: 125-2H) were from MBL (Woburn, MA). Penicillin, streptomycin, elastase, elastase inhibitor MeOSuc-Ala-Ala-Pro-Val chloromethyl ketone (MeOSu-AAPV-CMK), granulocyte elastase substrate (Glp-Pro-Val-pNA), Fluoromount G and monensin, were purchased from Sigma-Aldrich (St. Louis, MO). Ac-Tyr-Val-Ala-Asp-AOM (YVAD-CMK) caspase-1 inhibitor IV and AEBSF (Pefabloc-SC) were purchased from Calbiochem (Switzerland). X-Vivo15 medium was purchased from Lonza (Köln, Germany). Cathepsin G and proteinase 3 were from Athens Research and Technology

(Athens, GA). Human alpha thrombin was from Enzyme Research Laboratories (South Bend, IN). HMBPP was obtained from Cayman Chemical (Ann Arbor, MI, USA).

γδ T cell purification and culture

Peripheral blood samples were obtained from healthy donors volunteers after institutional Ethical Committee approval. Donors provided written informed consent before the collection of the samples. Peripheral blood mononuclear cells were isolated by standard density gradient centrifugation on Ficoll-Hypaque. Then, γδ T cells were purified by using magnetic cell sorting with the anti-TCR γ/δ MicroBead isolation kit, according to the manufacture's recommendations. The purity of recovered cells was higher than >98% in all the experiments as measured by flow cytometry. Cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and penicillin (100 U/ml), streptomycin (100 µg/ml).

Neutrophil purification and culture

Neutrophils were isolated from heparinized human blood samples by Ficoll-Hypaque gradient centrifugation and dextran sedimentation. Contaminating erythrocytes were removed by hypotonic lysis. After washing, cell pellets were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and penicillin (100 U/ml), streptomycin (100 µg/ml). The purity was checked by flow cytometry (>98%).

Immunostaining and flow cytometry

γδ T cells were stained with PE.Cy5-conjugated antibodies directed to CD69 or PE-conjugated anti-PAR1. In all cases, isotype matched control antibodies were used, and a gate based on size was defined in the analysis to exclude neutrophils. Neutrophil activation was evaluated by using a PE-conjugated antibody directed to CD11b. A gate based on size was done in the analysis to exclude γδ T cells. In all cases, analysis was performed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA).

Detection of cytokines by ELISA

γδ T cells ($1x10^6$/ml) were stimulated or not with anti-CD3 antibodies (250 ng/ml, 30 min at 37°C). Then, cells were cultured with or without neutrophils at a cell-to-cell ratio of 1:1. After 24 h of culture, supernatants were harvested and the presence of IFN-γ, TNF-α and IL-18 was analyzed by ELISA, according to the manufacturer's recommendations.

Elastase measurement

The elastase activity was determined by spectrophotometry from their ability to cleave a specific substrate. Briefly, neutrophils ($1x10^6$/ml) were cultured with or without γδ T cells at a cell-to-cell ratio of 1:1. After incubation, supernatants were recovered and incubated with elastase substrate, Glp-Pro-Val-pNA (1 nM) during 24 h at 37°C. Elastase concentration was determined by reading changes in optical density at dual wavelength 405-550 nm by spectrophotometry (Biochrom Asys UVM 340 Microplate Reader. Holliston, MA) and by interpolation in a standard elastase concentration curve.

Transwell co-culture

γδ T cells ($1x10^6$/ml) were stimulated with anti-CD3 antibodies (250 ng/ml, 30 min at 37°C) immobilized on the lower chamber of a 96-transwell plate with a polycarbonate filter of 0.4 μm pore size (Corning, MA, USA). Afterward, neutrophils were added in the upper chamber at γδ T cell:neutrophil ratio of 1:1. After 24 h of culture at 37°C, supernatants were recovered and the presence of IFN-γ and TNF-α was analyzed by ELISA, according to the manufacturer's recommendations.

Analysis of cell conjugates

Anti-CD3 antibodies (250 ng/ml) were immobilized on poly-L-lysine-coated glass coverslips (12 mm) (overnight at 37°C), after that the coverslips were washed with PBS, and γδ T cells were seeded and incubated at 37°C and 5% CO2 for 60 min. Then, neutrophils were added on coverslip at a cell-to-cell ratio of 1:1, and incubated for 2 h at 37°C. After incubation, the coverslips were carefully washed with PBS and cells were fixed in 2% paraformaldehyde and stained for elastase. Briefly, after fixation samples were permeabilized with 0.5% Triton X-100 in PBS for 1 min and blocked with 5% goat serum for 60 min at 37°C. Then samples were incubated with a rabbit anti-human elastase antibody (1 μg/ml) or the corresponding isotype controls for 45 min at 4°C. After that, cells were washed with PBS-BSA 0.25% and incubated with a DyLight549-goat anti-rabbit IgG antibody (9 μg/ml) for 30 min at 4°C. After wash, coverslips were mounted onto glass slides using Fluoromount-G solution. Immunofluorescence images were acquired with a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) using a Plapon 60 × 1.42 NA oil immersion objective. Images were analyzed using the Olympus FV10-ASW and Fiji software (National Institutes of Health).

Measurement of ROS production

Neutrophils were suspended in culture medium and incubated with 0.1 µg/ml DHR for 5 min at 37°C. Then, they were cultured with γδ T cells previously stimulated with HMBPP (10 µM, 90 min) or with anti-CD3 antibodies (500 ng/ml, 5 h) at a cell-to-cell ratio of 1:1. After 20 min at 37°C, cells were analyzed by flow cytometry by analyzing the variation of the MFI emission of DHR. A gate based on size was done in the analysis to exclude γδ T cells.

CD107a expression

γδ T cells were stimulated with HMBPP (10 µM) or anti-CD3 antibodies (500 ng/ml) during 1 h, then were cultured in presence of PE-conjugated anti-CD107a antibodies and monensin (2 µM) for 5 h at 37°C. After that, cells were washed and analyzed by flow cytometry. A gate based on size was done in the analysis to exclude neutrophils. As positive control, γδ T cells were incubated with PMA (50 ng/ml) and ionomycin (5 µg/ml). Results are expressed as percentage of positive cells for CD107a.

Statistical analysis

Student paired *t* test was used to determine the significance of differences between treatment groups. Multiple analyses were followed by Kruskal-Wallis's and Friedman's multiple-comparison post-test. The p values<0.05 were considered statistically significant.

Results

Neutrophils potentiate the activation of γδ T cells

Ex vivo manipulation and expansion of γδ T cells are key steps for successful adoptive transfer immunotherapies. Protocols has been recently developed for large-scale expansion of γδ T cells *ex vivo*, using immobilized anti-CD3 antibodies.[30,31] γδ T cells generated under these conditions must be tested for their immune capacity and for their effector function profiles. Taken this into account, we decided to evaluate the phenotype and function of γδ T cells activated by anti-CD3 antibodies and their interaction with neutrophils. For this purpose, freshly purified human γδ T cells were stimulated with immobilized anti-CD3 antibodies for 24 h. Then, we analyzed the expression of CD69 and the cytokine production. As expected, CD3 stimulation increased the expression of CD69 (Fig. 1a) and the production of the inflammatory cytokines IFN-γ (Fig. 1b) and TNF-α (Fig. 1c). To determine whether neutrophils were able to regulate the activation of γδ T cells, both cell types were co-cultured for 24 h at a cell-to-cell ratio of 1:1, according to previously settings established by our group.[29] In co-cultures, the viability of γδ T cells was not altered by incubating cells with neutrophils, as revealed by propidium iodide staining and flow cytometry analysis (data not shown). We observed that neutrophils potentiated the up-regulation of CD69 expression in γδ T cells (Fig. 1d) and the production of IFN-γ (Fig. 1e) and TNF-α (Fig. 1f). Of note, the production of these cytokines by neutrophils cultured alone was negligible (data not shown). Similarly to the effect we reported by employing HMBPP,[29] we found that γδ T cells activated by anti-CD3 antibodies also induced neutrophil activation as indicated by the up-regulation of CD11b and the release of elastase to the extracellular medium by neutrophils (Fig. 1g,h).

Soluble factors released by neutrophils regulate the activation of γδ T cells

We then speculated that soluble factors released by neutrophils activated by the co-culture with stimulated γδ T cells might be responsible for the increase in γδ T cell activation. First, we performed experiments using 96-transwell chambers with a polycarbonate filter (0.4 µm pore size). For this purpose, γδ T cells were included in the lower chamber of the transwell system previously coated with anti-CD3 antibodies, while neutrophils were seeded in the upper chamber at a γδ T cell:neutrophil ratio of 1:1. We observed that neutrophils enhanced the activation of γδ T cells even when both cell types were placed in different chambers of the transwell system, indicating that potentiation involves the participation of soluble factors released by neutrophils (Fig. 2a,b). Thus, to get insight into the neutrophil molecules involved in the activation of γδ T cells, we searched for factors secreted by activated neutrophils which could play a role in the activation of T cells. Among these molecules, IL-18 is an inflammatory cytokine produced by neutrophils in response to stimulation by TNF-α[32] that promotes both the proliferation of CD8+ T cells stimulated by anti-CD3 antibodies,[33] and the expansion of NK cells in the presence of IL-2.[34] Moreover, it was reported that IL-18 stimulates the proliferation of γδ T cells activated by IL-2 and zoledronate, and the blockade of IL-18Rα chain strongly inhibits the proliferation of γδ T cells, suggesting the involvement of IL-18 signaling.[35] Then, we decided to determine whether IL-18 was responsible of neutrophil enhancement of γδ T cell activation induced by anti-CD3 stimulation. However, we detected low levels of IL-18 in supernatants obtained from co-cultures of γδ T cells and neutrophils in presence of anti-CD3 antibodies (Fig. 2c). Because we could not exclude that IL-18 produced by neutrophils could act on γδ T cells even if it was produced at low concentrations, we performed experiments using a

specific inhibitor of caspase-1, YVAD-CMK, to inhibit the maturation of pro-IL-18 to IL-18. Co-culture of γδ T cells and neutrophils in the presence of YVAD-CMK did not abrogate the increase in γδ T cell activation induced by neutrophils as measured by either CD69 expression and IFN-γ and TNF-α production (Fig. 2d-f). Of mention, YVAD-CMK was able to inhibit neutrophil IL-1β secretion, another cytokine which relies on caspase-1 to generate its mature form[36] (see Supplementary material, Fig. S1). We also performed blocking experiments by adding neutralizing antibodies anti-IL-18 to neutrophil-γδ T cells co-cultures, and we observed that this treatment did not inhibit γδ T cells stimulation induced by neutrophils (see Supplementary material, Fig. S2a-c), confirming that IL-18 does not play a role in the potentiation of γδ T cell activation induced by neutrophils. It has also been reported that neutrophil proteases could regulate γδ T cell function.[37] As we determined that upon co-culture with activated γδ T cells, neutrophils release elastase (Fig. 1h), we speculated that this serine protease could modulate γδ T cell function. To test this hypothesis we used the irreversible inhibitor of serine proteases AEBSF (Pefabloc-SC). This compound abrogated the increase in γδ T cell activation induced by neutrophils (Fig. 2g-i). In line with this findings, elastase added to γδ T cell cultures in absence of neutrophils, reproduced the increase in the production of IFN-γ (Fig. 3a) and TNF-α (Fig. 3b) observed in co-cultures of γδ T cells and neutrophils. A similar effect was observed in cells treated with cathepsin G and proteinase 3 (see Supplementary material, Fig. S3a-c). Furthermore, when we pre-incubated elastase with its specific inhibitor MeOSu-AAPV-CMK, the effect of this serine protease was reverted, demonstrating that its action on γδ T cells rely on the enzymatic activity (Fig. 3c). We hypothesizes that polarized release of elastase from neutrophils to γδ T cells would potentiate γδ T cell function, and to evaluate this, we

performed fluorescence microscopy studies. Interestingly, the results showed that neutrophils actually interact with γδ T cells (Fig. 3d), and a quantitative analysis revealed that when cultured together for 2 h at 37°C, 32 ± 1% of neutrophils formed cell conjugates with γδ T cells (mean ± SEM, n= 2). Among conjugates, there was a 69 ± 1% of them expressing elastase in the interface between γδ T cells and neutrophils (Fig. 3e). Moreover, the fluorescence intensity was higher at the site of cell contact (Fig. 3f,g). Additionally, when we performed co-culture of γδ T cells with neutrophils in presence of elastase inhibitor, we reverted completely the potentiation of CD69 expression on γδ T cells (Fig. 3h), and partially the effect on cytokine production (Fig. 3i,j). This partial reduction observed on cytokine secretion could be due to the fact that low concentrations of elastase inhibitor had to be used because at higher concentrations this inhibitor exhibited off target effects on γδ T cells themselves (data not shown).

PAR1 modulates γδ T cell activation induced by CD3 stimulation

In different cell types, serine proteases induce cell activation by acting on PARs. These receptors are G-protein-coupled receptors expressed on cell surface, and are activated by irreversible proteolytic cleavage, leading to the exposure of a cryptic amino-terminus sequence, which serves as a tethered ligand. The newly exposed tethered ligand then activates the receptor,[20] and this self-activation prompts the conformational change of the receptor that allows interactions with G proteins.[38] In humans, there are four PARs described to date: PAR1, PAR2, PAR3 and PAR4, which are expressed by different cell types including circulating blood cells.[21-24] Recently it was reported that γδ T cells only express PAR1.[39] Since this receptor is activated by elastase among other proteases, we decided to evaluate the role of PAR1 during γδ T cell stimulation by neutrophils. First,

we analyzed the expression of this receptor on purified γδ T cells employing B cells as a negative control. As shown in Fig. 4(a-c), γδ T cells express PAR1. This receptor was functional as we could activate it by thrombin, a specific agonist. Thrombin treatment increased CD69 expression (Fig. 4d) and cytokine production (Fig. 4e,f) by γδ T cells. Then, we decided to examine the effect of blocking PAR1 with the specific antagonist RWJ56110. For this purpose, γδ T cells were pretreated with this compound and then stimulated with anti-CD3 in the presence or absence of neutrophils. As shown in Fig. 4(g-i), RWJ56110 reverted the potentiation of γδ T cell activation induced by neutrophils, as measured through the expression of CD69 (Fig. 4g), and the production of IFN-γ (Fig. 4h) and TNF-α (Fig. 4i).

Elastase and reactive oxygen species during γδ T cells activation

We have been previously reported[29] that neutrophils inhibited the activation of γδ T cells induced by HMBPP by a mechanism dependent on the production of ROS. To study the contrasting effects exerted by neutrophils on HMBPP and anti-CD3 stimulated γδ T cells, we decided to analyze the release of elastase by neutrophils co-cultured with γδ T cells activated by HMBPP, as well as the production of ROS when neutrophils were co-culture with γδ T cells activated with anti-CD3 antibodies. Fig. 5(a) shows that in presence of γδ T cells activated with HMBPP, neutrophils release elastase. Moreover, in presence of the elastase inhibitor, the reduction mediated by neutrophils of γδ T cell activation induced by HMBPP, was even higher compared to that observed in absence of the inhibitor (Fig. 5b,c). In line with these results, we observed that the addition of serine proteases elastase, cathepsin G and proteinase 3 increased the activation of γδ T

cells induced by HMBPP (see Supplementary material, Fig. S4a,b). These findings suggest that upon HMBPP activation, the inhibitory role of ROS and the enhancer effect of elastase coexist, and when elastase is inhibited, ROS are more efficient to suppress γδ T cell activation. On the other hand, in contrast to that observed when neutrophils are cultured with γδ T cells activated by HMBPP, we did not detect ROS production when these cells were activated by anti-CD3 (Fig. 5d,e). These results suggest that ROS are not involved in the regulation of γδ T cell activation by anti-CD3 antibodies. Interestingly, in additional assays, we observed that γδ T cells activated through CD3 showed higher cytotoxic activity compared to γδ T cells stimulated by HMBPP or control cells (Fig. 5f,g), further supporting that different stimuli can induce distinct profiles of activation.

Discussion

Neutrophils produce a broad array of molecules[11] that contribute to the modulation of function of immune cells such as macrophages, dendritic cells, NK cells, and T cells.[29,40-43] In this work, we show for the first time that freshly isolated neutrophils potentiate the activation of γδ T cells stimulated through CD3. We observed that neutrophils increased the up-regulation of CD69 and the production of IFN-γ and TNF-α by anti-CD3-stimulated γδ T cells. IL-18 is a proinflammatory cytokine secreted by neutrophils, and it was reported that it can enhance the activation of T cells.[33,35] However, our results showed that IL-18 is not involved in the potentiation of γδ T cell activation by neutrophils. A possible role for this cytokine was ruled out because inhibition of caspase-1, the enzyme that processes pro-IL-18 into IL-18, and blocking experiments using anti-IL-18 neutralizing antibodies did not impede the potentiation of

γδ T cell activation by neutrophils. By contrast, an inhibitor of serine proteases was able to inhibit the increase in γδ T cell activation mediated by neutrophils; and the addition of exogenous elastase could reproduce neutrophil effects, suggesting that this enzyme released by neutrophils, upon co-culture with activated γδ T cells, promotes further γδ T cell activation. Previous studies showed that neutrophil serine proteases can cleave the N-terminal extracellular domain of PARs.[38] We here demonstrated that γδ T cell stimulation by neutrophil elastase was mediated through PAR1, since inhibition of this receptor abrogated the potentiation of γδ T cell activation mediated by neutrophils. Our results are in accordance with those previously reported by Hurley and co-workers, which showed that activation of PAR1 by thrombin in conventional T cells (αβTCR), promotes antigen-dependent cytokine production and tissue inflammation.[44]

Noteworthy, Fazio and co-workers showed in neutrophil-free γδ T cell cultures, that treatment of cells with serine proteases inhibits γδ T cell proliferation and effector functions induced by phosphoantigens.[37] Contrasting with these findings, we also observed a stimulatory effect of proteases on HMBPP-stimulated γδ T cells. The reasons for this discrepancy remain uncertain, but may be related to differences in the experimental setting between both works. In fact, studies conducted by Fazio and co-workers used γδ T cells amplified from peripheral mononuclear cells stimulated by zoledronate and IL-2, while we employed magnetic bead-purified γδ T cells stimulated by the exogenous phosphoantigen HMBPP.

We previously reported that ROS play a major role in the down modulation of γδ T cell activation induced by HMBPP.[29] In contrast to those findings, we showed here that when γδ T cells are stimulated through CD3 the production of ROS was undetectable,

and elastase activity was responsible of the modulation of T cell function by neutrophils. These results suggest that different stimuli induce diverse activation states of γδ T cells that allow them to respond differentially to extracellular factors. In this regard, it has been recently reported that stimulation by anti-CD3 antibodies triggers conformational changes in the human Vγ9Vδ2 TCR.[45] Moreover, different anti-CD3 antibodies, such as UCHT1 and OKT3 promotes differential responses on γδ T cells, being UCHT1 antibodies more potent to prompt CD3 conformational changes than OKT3.[45] Furthermore, γδ T cells, not only display differences at the CD3 conformation, but also they show differences in the intracellular signaling downstream of the TCR. Thus, triggering conformational changes in CD3 in the γδ TCR led to enhanced signaling events.[45] More interesting, Dopfer and co-workers reported that stimulation of γδ T cells by the synthetic phosphoantigens, bromohydrin pyrophosphate or isopentenyl pyrophosphate do not induce the conformational changes in CD3, demonstrating a differential behavior of γδ T cells when they are stimulated with different agonists. These findings could explain the dissimilarities in the degranulation capacity that we observed in γδ T cells stimulated with HMBPP or with anti-CD3 antibodies. In this regard, we observed that stimulation through CD3 promotes higher levels of degranulation than that induced by HMBPP as judged by an increased CD107a expression on γδ T cells.

It is important to consider that the activity of γδ T cells against target cells depends, not only on the expression of cognate antigens, but also on the resulting balance among signals from costimulatory molecules, adhesion molecules and activating or inhibitory NK receptors.[46,47] Thus, it is possible to speculate that depending of the resulting signal, γδ T cell differentially interact with neutrophils, modulating ROS production levels

which would in turn shift the balance towards inhibition or upregulation of γδ T cell activation. This could also explain the inhibitory effect of serine proteases reported by Fazio and co-workers, on γδ T cell proliferation induced by particles coated with anti-CD2, anti-CD3 and anti-CD28 antibodies,[37] which constitute a multiple activator signal. Taking together, this set of circumstances allows us to propose that γδ T cells could respond differentially to neutrophil regulation if they are activated by different stimuli, such as phosphoantigens or anti-CD3 antibodies, as we demonstrated in our present work. γδ T cell physiology is complex and its activation could have many levels of regulation.

Besides the role of serine proteases during innate immune responses, it was observed in different lung diseases, that the initiation and propagation of lung damage is a consequence of an exaggerated inflammatory response, which includes the release of proteases and cytotoxic products by leukocytes.[16,48] A balance between proteases and anti-proteases is required, and when proteases are not neutralized properly it results in lung damage.[49] To control exacerbated proteolysis at sites of inflammation, the administration of therapeutic inhibitors of proteases has been tested as a therapy for a variety of inflammatory and infectious lung diseases.[50] In pathologies such as chronic obstructive pulmonary disease, γδ T cells are present in the inflamed tissue, and they are an important source of IL-17A, which is a cytokine that regulates lung immunity and inflammation.[51] Taken this into account, we speculate that the inhibition of serine proteases (i.e. elastase) would reduce not only their own effect but also the action of the enzyme on γδ T cells, thereby limiting a harmful immune response mediated by these T cells.

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Figure legends

Figure 1. Neutrophils potentiate the activation of γδ T cells. γδ T cells were incubated during 30 min on 96-flat bottom culture plates, previously coated with anti-CD3 antibodies (250 ng/ml). (**A-C**) After 24 h of culture, the expression of CD69 (n= 14) (A) and the production of IFN-γ (n= 18) (B) and TNF-α (n= 14) (C) by γδ T cells were analyzed. The data represent the mean ± SEM. ***p<0.001, Wilcoxon matched ranked two-tailed test. (**D-H**) After 30 min of incubation, neutrophils were added to γδ T cell culture at a cell-to-cell ratio of 1:1 and incubated during 24 h. Then, the expression of CD69 (n= 15) (D) and the production of IFN-γ (n= 24) (E) and TNF-α (n= 11) (F) by γδ T cells were analyzed. The data represent the mean ± SEM. *p<0.05 vs stimulated-γδ T cells in absence of neutrophils (Ne), Friedman test for multiple comparisons with Dunn's posttest. Additionally, the expression of CD11b by neutrophils was analyzed by flow cytometry (G) and the release of elastase was quantified in cell supernatants by using the granulocyte elastase substrate (Glp-Pro-Val-pNA) and spectrophotometry analysis (H). The data represent the mean ± SEM, n= 4 (CD11b) and n= 6 (elastase). *p<0.05, Mann-Whitney one-tailed test.

Figure 2. Soluble factors are involved during activation of γδ T cells by neutrophils. (**A** and **B**) γδ T cells were stimulated with anti-CD3 antibodies (250 ng/ml) previously immobilized on the lower compartment of a 0.4 µm pore size membrane transwell system. After 30 min, neutrophils were seeded (+Ne) or not (-Ne) in the upper chamber at a γδ T-cell:neutrophil ratio of 1:1. After 24 h the production of IFN-γ and TNF-α was analyzed by ELISA. Data are shown as mean ± SEM, n= 4.

*p<0.05, Mann-Whitney two-tailed test. (**C**-**I**) γδ T cells were incubated during 30 min on 96-flat bottom culture plates, previously coated with anti-CD3 antibodies (250 ng/ml). Then, neutrophils were added at a cell-to-cell ratio of 1:1, and incubated during 24 h. (**C**) After incubation, supernatants were recovered and IL-18 was measured by ELISA. The data represent the mean ± SEM, n= 4. *p<0.05 vs stimulated γδ T cells in absence of Ne, Kruskal-Wallis test for multiple comparisons with Dunn's posttest. (**D**-**F**) Neutrophils were pre-incubated with caspase-1 inhibitor (YVAD) (20 μM, 30 min at 37°C), then were added to γδ T cell culture, the inhibitor was present during the whole incubation time. After 24 h of incubation, the expression of CD69 (n= 7) (D), and the production of IFN-γ (n= 5) (E) and TNF-α (n= 3) (F) by γδ T cells were analyzed. The data represent the mean ± SEM. *p<0.05 vs stimulated γδ T cells in absence of Ne, Friedman test for multiple comparisons with Dunn's posttest. (**G-I**) Neutrophils were pre-incubated with the irreversible serine proteases inhibitor, Pefabloc-SC (PF, 20 μg/ml, 30 min at 37°C). The inhibitor was present during the whole incubation time. The expression of CD69 (n= 13) (G), and the production of IFN-γ (n= 7) (H) and TNF-α (n= 4) (I) by γδ T cells were analyzed. The data represent the mean ± SEM. *p<0.05 vs stimulated γδ T cells in absence of Ne without PF and #p<0.05 vs stimulated γδ T cells in presence of Ne without PF, Friedman test for multiple comparisons with Dunn's posttest. n.s.= non-significant.

Figure 3. Elastase activates γδ T cells. (**A** and **B**) γδ T cells were incubated during 30 min on 96-flat bottom culture plates, previously coated with anti-CD3 antibodies (250 ng/ml). After incubation, neutrophils were added at a cell-to-cell ratio of 1:1, and cultured during 24 h. Then, the secretion of IFN-γ (n= 5) (A) and TNF-α (n= 6) (B) was

analyzed in cell supernatants. The data represent the mean ± SEM. * and #$p<0.05$ vs γδ T cells alone, Kruskal-Wallis test for multiple comparisons with Dunn's posttest. (**C**) Elastase was pre-incubated with the elastase inhibitor (EI, 3 μM) during 30 min at 37°C, previously to be added to anti-CD3 activated-γδ T cells. Then, cells were cultured for 24 h and IFN-γ secretion was analyzed. The data represent the mean ± SEM, n= 3. *$p<0.05$ vs γδ T cells alone and # $p<0.05$ vs γδ T cells stimulated by ELA, Friedman test for multiple comparisons with Dunn's posttest. (**D-G**) γδ T cells were seeded on anti-CD3 coated coverslips and incubated for 60 min at 37°C, then neutrophils were added at a cell-to-cell ratio of 1:1. After 2 h at 37°C coverslips were carefully washed, cells were fixed, permeabilized and stained with anti-elastase antibodies. The formation of cell conjugates and the distribution of neutrophil elastase were analyzed by microscopy. (D) Figure shows representative images of cell conjugates, elastase is shown in red. For the experiment shown, 340 cells were analyzed. Bar: 5 μm. (E) Represents the percentage of cell conjugates in which elastase was recruited at the cell interaction site (ELA+) or in which elastase was absent of this site (ELA-). (F) Histogram shows the intensity of elastase detected at the cell-to-cell contact area (contact) or in the opposite site of interaction area (no contact), expressed as arbitrary units (AU). *$p<0.05$, Wilcoxon matched ranked two-tailed test. (G) Representative diagram of elastase intensity in contact vs no contact area. This analysis was performed by drawing a line in the contact and no contact zone, arbitrarily defined, and by analyzing the fluorescence intensity following the lines using the Fiji software. (**H-J**) Previously to be added to γδ T cell culture, neutrophils were incubated with the elastase inhibitor (EI, 3 μM, 30 min at 37°C). The inhibitor was present during the whole incubation time. The expression of CD69 (n= 4) (H), and the production of IFN-γ (n= 5) (I) and TNF-α (n= 5) (J) by γδ T

cells were analyzed. The data represent the mean ± SEM. *p<0.05 and **p<0.001 vs stimulated γδ T cells in absence of Ne without EI, #p<0.05 vs stimulated γδ T cells in absence of Ne with EI. Friedman test for multiple comparisons with Dunn's posttest. n.s.= non-significant.

Figure 4. PAR1 mediates the effect of neutrophil elastase on γδ T cells activation. (**A** and **C**) γδ T cells were stained with anti-PAR1 antibodies and analyzed by flow cytometry. (A) Histograms of PAR1 expression on γδ T cells. Figure shows a representative experiment out of 12 performed. B cells were employed as negative control for PAR1 expression. Black thin line: isotype control, gray thick line: PAR1 expression. (B) Shows representative image of PAR1 expression on γδ T cells. Bar: 5 μm. (C) Percentage of PAR1 expression on γδ T cells, after subtracting the isotype control. (**D-F**) γδ T cells were incubated during 30 min on 96-flat bottom culture plates, previously coated with anti-CD3 antibodies (250 ng/ml). Then, cells were culture with or without thrombin (TRB, 10 U/ml) for 24 h. The expression of CD69 (n= 13) (D), and the production of IFN-γ (n= 7) (E) and TNF-α (n= 7) (F) by γδ T cells were analyzed. The data represent the mean ± SEM. *p<0.05, Mann-Whitney two-tailed test. (**G-I**) γδ T cells were incubated during 30 min with or without the PAR1 antagonist, RWJ56110 (RWJ, 3 μM), then cells were seeded on 96-flat bottom culture plates, previously coated with anti-CD3 antibodies (250 ng/ml). After 30 min of incubation, neutrophils were added at a cell-to-cell ratio of 1:1 and incubated for additional 24 h. The antagonist was present during the whole incubation time. The expression of CD69 (n= 6) (G), and the production of IFN-γ (n= 4) (H) and TNF-α (n= 8) (I) by γδ T cells were analyzed. The

data represent the mean ± SEM. *p<0.05 vs stimulated γδ T cells in absence of Ne, Friedman test for multiple comparisons with Dunn's posttest. n.s.= non-significant.

Figure 5. Elastase and reactive oxygen species as modulators during γδ T cell activation. γδ T cells were stimulated with HMBPP (10 μM, 60 min at 37°C), then washed and culture with neutrophils at a cell-to-cell ratio of 1:1. After 24 h, the release of elastase was quantified in cell supernatants by using the granulocyte elastase substrate (Glp-Pro-Val-pNA) and spectrophotometry analysis (**A**). The data represent the mean ± SEM, n= 9. **p<0.01, Wilcoxon two-tailed test. (**B** and **C**) Previously to be added to γδ T cells culture, neutrophils were incubated with the elastase inhibitor (EI, 3 μM, 30 min at 37°C). The inhibitor was present during the whole incubation time. The production of IFN-γ (B) and TNF-α (C) by γδ T cells were analyzed. The data represent the mean ± SEM, n= 10. *p<0.05, **p<0.01 and ***p<0.001, Kruskal-Wallis test for multiple comparisons with Dunn's posttest. (**D** and **E**) Neutrophils loaded with DHR were incubated with or without γδ T cells activated or not with HMBPP (10 μM) or with anti-CD3 antibodies (500 ng/ml) at a cell-to-cell ratio of 1:1, during 20 min at 37°C. Then, cells were analyzed by flow cytometry. (D) Histograms are from a representative experiment of four performed. Gray histogram: neutrophils not loaded with DHR (negative); thin line histogram: neutrophils loaded with DHR cultured alone; dotted line histogram: neutrophils loaded with DHR cultured with non-stimulated γδ T cells; thick line histogram: neutrophils loaded with DHR and incubated with HMBPP-stimulated γδ T cells; and stripy line histogram: neutrophils loaded with DHR incubated with anti-CD3-stimulated γδ T cells. (E) Percentage of neutrophils that emitted fluorescence over an arbitrary cut-off established with unstimulated neutrophils.

***p<0.001 compared to Ne alone, Kruskal-Wallis test for multiple comparisons with Dunn's posttest. (**F** and **G**) γδ T cells were stimulated with HMBPP (10 µM, 60 min at 37°C) or anti-CD3 antibodies (500 ng/ml, 60 min at 37°C), then washed and cultured with neutrophils at a cell-to-cell ratio of 1:1. After 5 h in presence of anti-CD107a antibodies and monensin, cells were washed and analyzed by flow cytometry. As positive control, cells were stimulated with PMA and ionomycin (P/I). (F) Representative density-plots, n= 10. (G) Percentage of positive γδ T cells for CD107a. The data represent the mean ± SEM, n= 10. ***p<0.001 and ****p<0.0001 vs control, Kruskal-Wallis test for multiple comparisons with Dunn's posttest.

Figure 1

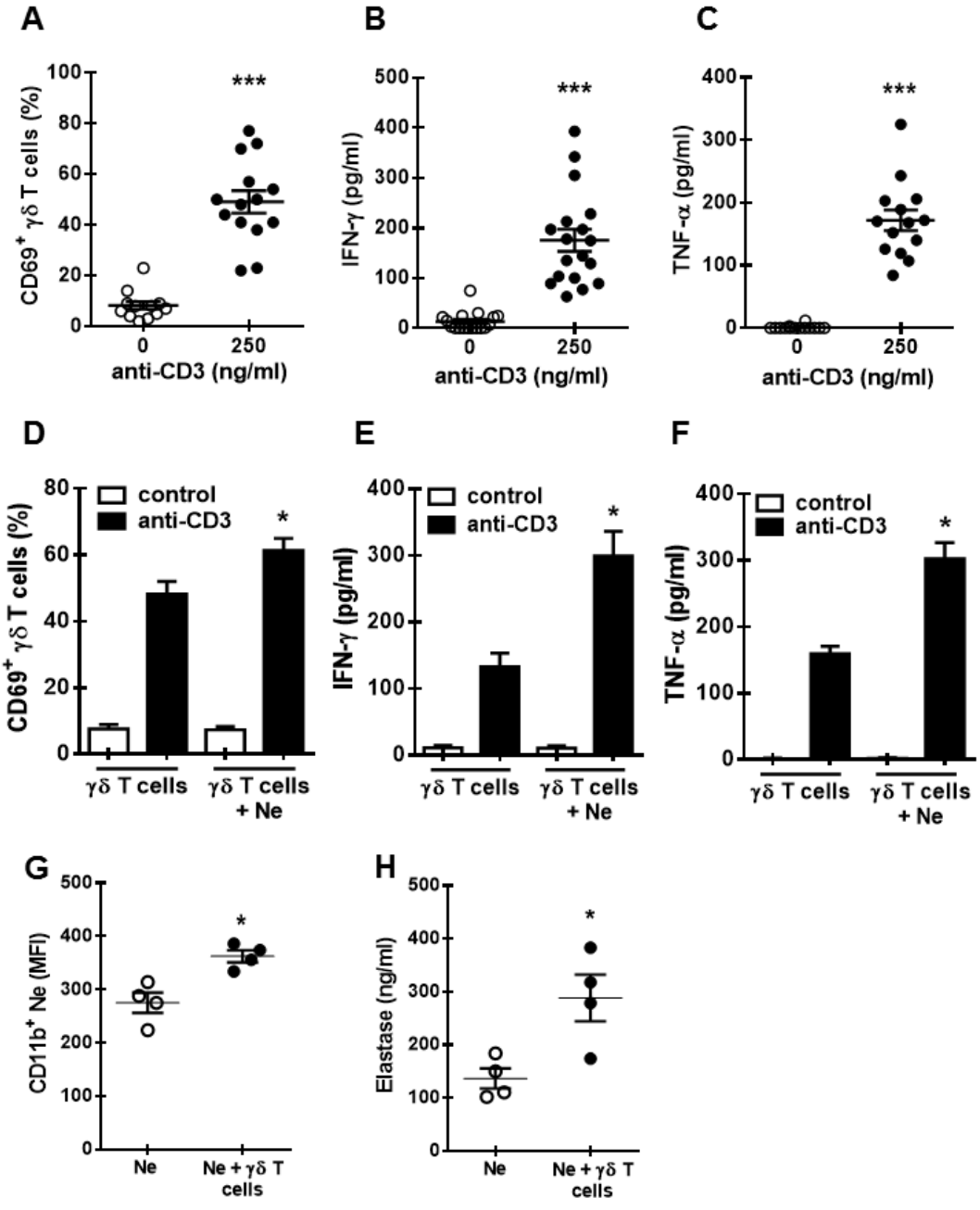

Figure 2

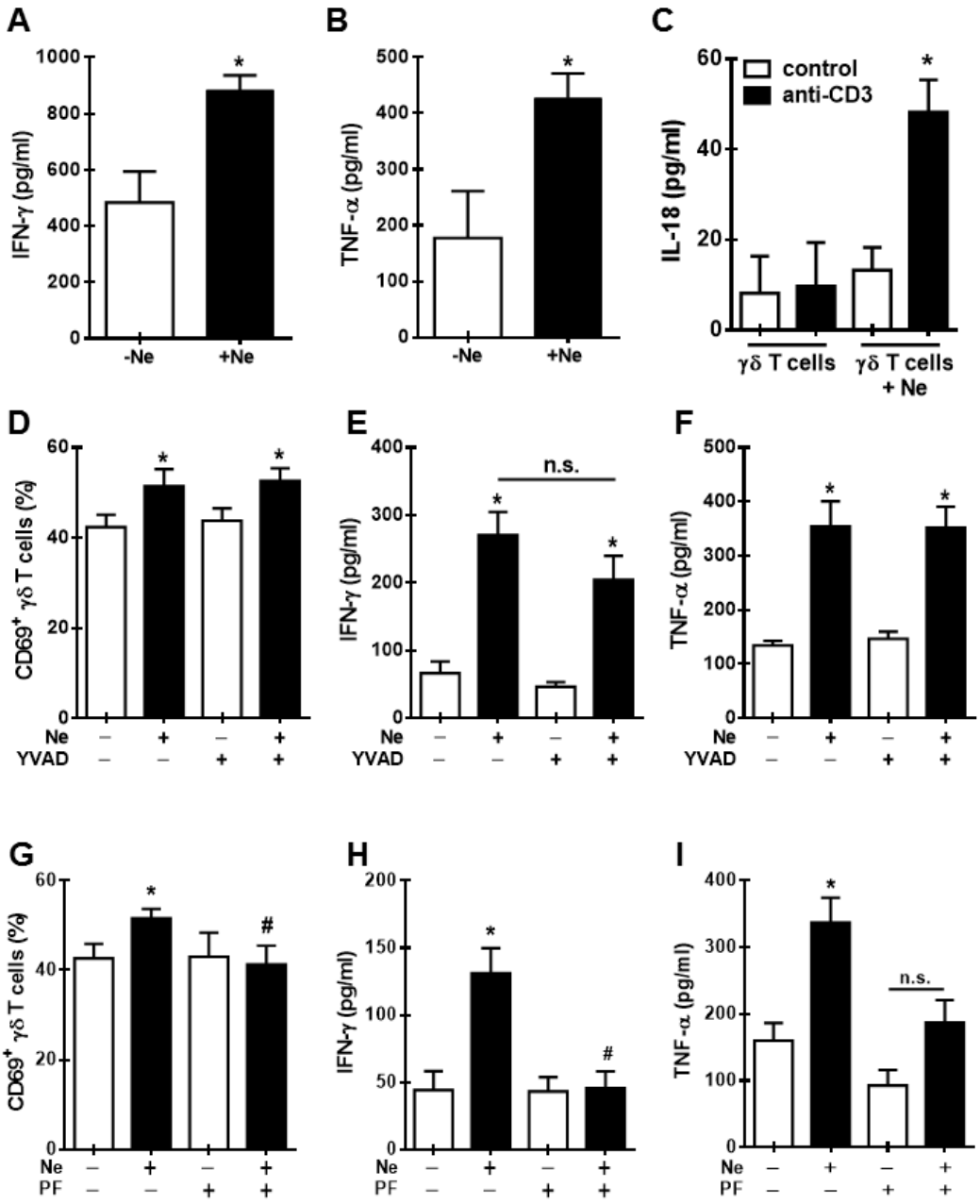

Figure 3

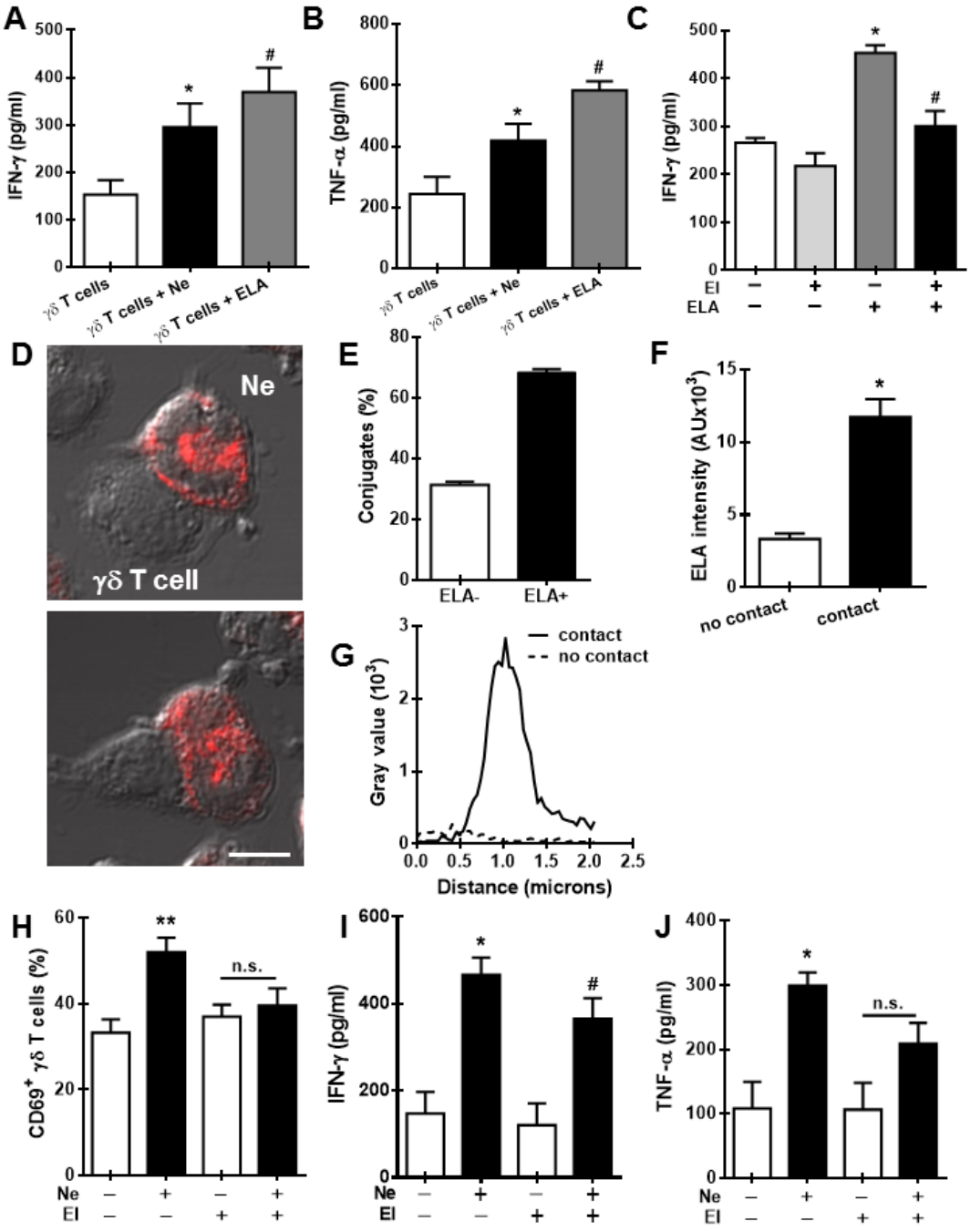

Figure 4

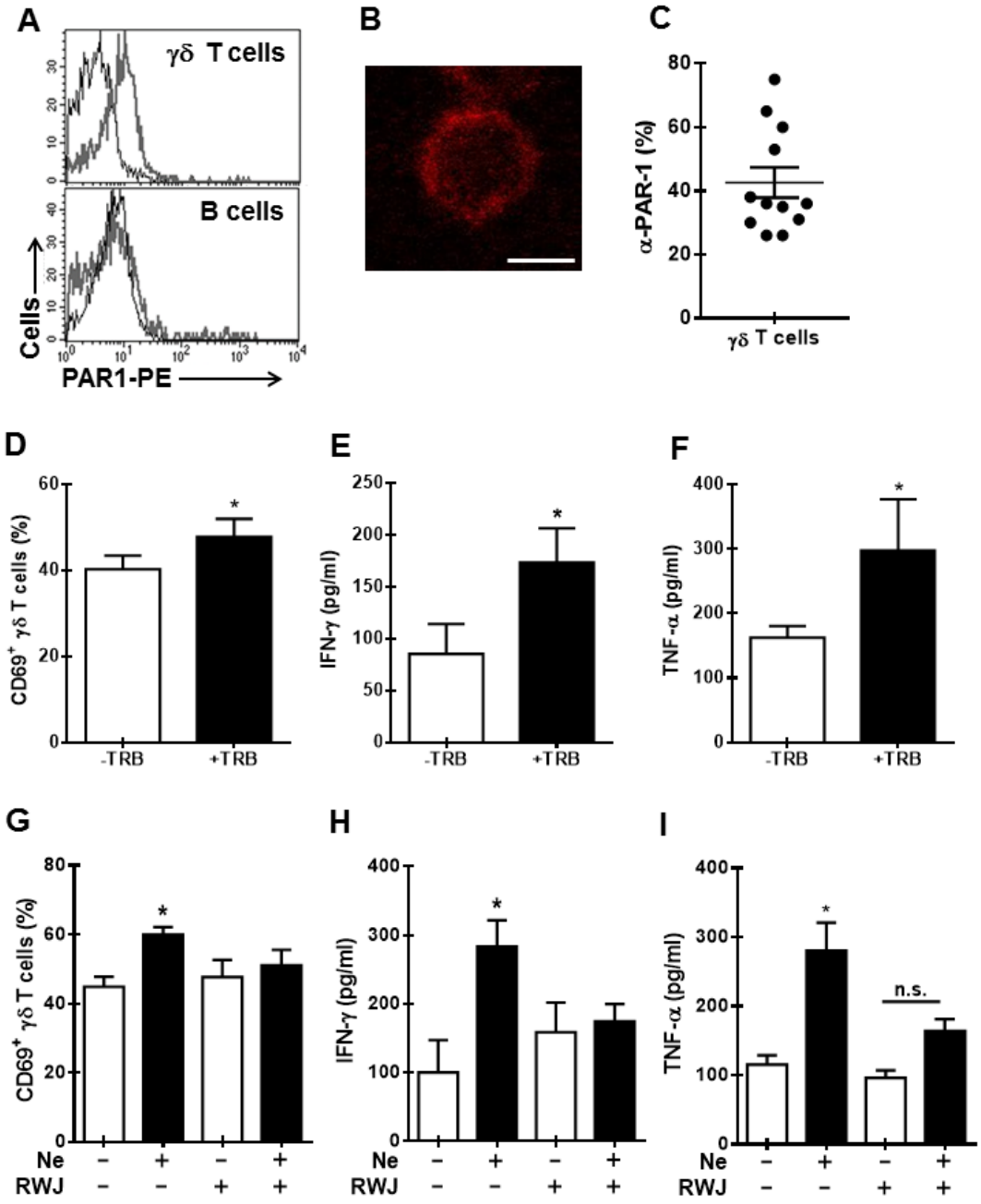

Figure 5