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Neutrophils suppress γδ T-cell function

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γδ T cells have been shown to stimulate the recruitment and activation of neutrophils through the release of a range of cytokines and chemokines. Here, we investigated the reverse relationship, showing that human neutrophils suppress the function of human blood γδ T cells. We show that the upregulation of CD25 and CD69 expression, the production of IFN-γ, and the proliferation of γδ T cells induced by (E)-1-hydroxy-2-methylbut-2-enyl 4-diphosphate are inhibited by neutrophils. Spontaneous activation of γδ T cells in culture is also suppressed by neutrophils. We show that inhibitors of prostaglandin E2 and arginase I do not exert any effect, although, in contrast, catalase prevents the suppression of γδ T cells induced by neutrophils, suggesting the participation of neutrophil-derived ROS. We also show that the ROS-generating system xanthine/xanthine oxidase suppresses γδ T cells in a similar fashion to neutrophils, while neutrophils from chronic granulomatous disease patients only weakly inhibit γδ T cells. Our results reveal a bi-directional cross-talk between γδ T cells and neutrophils: while γδ T cells promote the recruitment and the activation of neutrophils to fight invading pathogens, neutrophils in turn suppress the activation of γδ T cells to contribute to the resolution of inflammation.

Keywords: γδ T cells · Neutrophils · ROS

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Introduction

γδ T cells constitute a functionally specialized subset of T lymphocytes that play an important role in linking the innate and adaptive immunity [1]. γδ T cells expressing the Vγ9Vδ2TCR are only found in higher primates and humans [2]. They represent the vast majority of γδ T cells in human peripheral blood [3]. In healthy adults, they comprise about 0.5–5% of circulating T cells [4], however, the number of Vγ9Vδ2 T cells can dramatically increase during the early response to many viral, bacterial, and parasitic infections, at times comprising up to >50% of all circulating T cells within a few days [5]. Vγ9Vδ2 T cells acquire a preactivated phenotype early in their development allowing the rapid induction of a wide variety of functions upon stimulation in a non-MHC restricted manner by phosphoantigens such as (E)-1-hydroxy-2-methylbut-2-enyl 4-diphosphate (HMBPP) [6–9]. These functions include a highly cytotoxic response against infected and transformed cells, the production of a range of cytokines and chemokines, the recruitment and the activation of neutrophils, the differentiation of

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monocytes into a proinflammatory profile, the phenotypic maturation of dendritic cells (DCs), the polarization of $CD4^+$ T cells into a Th1 profile, the promotion of B-cell activation, and the presentation of antigenic peptides to both $CD4^+$ and $CD8^+$ T cells [10–15].

Neutrophils play a critical role in the immune response against bacteria and fungi infection. They ingest and destroy microbes through the action of a large array of antimicrobial weapons, which include ROS, proteolytic enzymes, and antimicrobial proteins [16, 17]. These toxic weapons do not discriminate self from nonself; hence they are also able to induce host tissue damage in different pathologic conditions [18]. Neutrophils are the first immune cells infiltrating the infected tissues and growing evidence supports a critical role of γδ T cells in the recruitment and activation of neutrophils at the sites of infection and inflammation [12, 19–24]. Moreover, Caccamo et al. [11] have recently shown that human Vγ9Vδ2 T cells cultured with phosphoantigens and a cocktail of Th17-promoting cytokines (IL-1β, TGF-β, IL-6, and IL-23) selectively express the transcription factor ROR-γt and produce IL-17. They also demonstrated that upon activation, $IL\text{-}17^+$ Vγ9Vδ2 T cells produce CXCL8, increasing the recruitment of neutrophils and stimulating their phagocytic ability [11]. On the other hand, Davey et al. [25] found that Vγ9Vδ2 T cells promote the inflammatory activity of neutrophils not only by inducing their activation but also by providing potent survival signals that rescued them from undergoing apoptosis.

In this study, we analyzed whether neutrophils were able to modulate the phenotype and function of human blood γδ T cells. We found that neutrophils effectively suppress the activation of γδ T cells. Our data demonstrate for the first time the existence of a bidirectional cross-talk between γδ T cells and neutrophils. While γδ T cells promote the recruitment and the activation of neutrophils to fight invading pathogens, the ability of neutrophils to inhibit the activation of γδ T cells might contribute to the resolution of inflammation and the restoration of tissue homeostasis.

Results

Neutrophils prevent the activation of γδ T cells

Freshly purified γδ T cells were obtained from peripheral blood of healthy donors and then stimulated with the agonist HMBPP (10 μM). The expression of the activation marker CD25 was analyzed 24 h later by flow cytometry. As expected, HMBPP stimulation increased the expression of CD25 (Fig. 1A). Unless otherwise stated, all the experiments directed to determine whether neutrophils were able to regulate the activation of γδ T cells were performed using autologous neutrophils. A representative dot plot showing the purity of neutrophils is illustrated in Figure 1B. Unstimulated or HMBPP-treated γδ T cells were cultured for 24 h with or without neutrophils and the expression of CD25 was analyzed by flow cytometry. Neutrophils effectively prevented the upregulation of CD25 expression in γδ T cells induced by either 10 or 50 μM HMBPP (Fig. 1C–E). The inhibitory effect mediated by neutrophils showed a high reproducibility between different donors (Fig. 1F). The upregulation of CD69 induced by HMBPP was also impaired in γδ T cells cultured with neutrophils (Fig. 1G). It should be noted that not only the activation induced by HMBPP but also the spontaneous activation of γδ T cells was significantly inhibited by neutrophils (Fig. 1D–G). The viability of γδ T cells cultured with or without neutrophils was in all cases higher than 90%, as revealed by PI staining and flow cytometry (data not shown).

To determine whether the inhibitory effect of neutrophils on the activation of γδ T cells was dependent on neutrophil viability, we analyzed whether apoptotic neutrophils prevented the activation of γδ T cells. To obtain apoptotic neutrophils, cells were cultured alone in protein-free medium for 18 h and apoptosis was then evaluated by fluorescence microscopy. The percentage of apoptotic neutrophils was in all cases higher than 80% (87 ± 7, mean ± SEM, $n = 7$). This cell population highly enriched in apoptotic neutrophils was co-cultured for 24 h with γδ T cells previously activated, or not, by HMBPP. As a control, γδ T cells were also co-cultured with freshly isolated neutrophils. Apoptotic neutrophils did not prevent the spontaneous or HMBPP-induced activation of γδ T cells, measured as the upregulation of CD25 and CD69 expression and the stimulation of IFN-γ production (Fig. 2A–C). As expected, fixed neutrophils were also unable to prevent the activation of γδ T cells (Fig. 2A–C). Further experiments were performed to evaluate whether other cell populations were able to prevent the activation of γδ T cells. To this aim, PBMCs previously depleted of γδ T cells were co-cultured with activated γδ T cells using a cell-to-cell ratio of 1:1. After 24 h, the expression of CD25 and CD69 by γδ T cells was analyzed. Figure 2D and E shows that mononuclear cells did not exert any inhibitory effect. To further characterize the impact of neutrophils on the function of γδ T cells, we analyzed whether the inhibitory effect of neutrophils was transient or irreversible. In these experiments, after incubation for 24 h with or without neutrophils, γδ T cells were purified again and cultured alone for an additional period of 24 h. Results in Figure 2F and G show that γδ T cells were unable to overcome the inhibition induced by the previous exposure to neutrophils.

We then analyzed whether neutrophils were able to modulate two additional functions of γδ T cells, the production of IFN-γ and cell proliferation. We found that the production of IFN-γ triggered by either 1 or 10 μM of HMBPP was markedly inhibited by neutrophils (Fig. 3A). A significant inhibition of γδ T-cell proliferation was also observed when cells were stimulated only by IL-2 as well as by IL-2 plus 1 μM of HMBPP. By contrast, no inhibition was observed using IL-2 plus 10 μM of HMBPP (Fig. 3B and C). This suggests that, at least for some responses mediated by γδ T cells, the suppressive effect of neutrophils could be overcome by increasing the concentration of the γδ T-cell stimuli.

Neutrophils suppress γδ T cells by ROS-dependent mechanism

A large body of evidence indicates that γδ T cells are able to induce the activation of neutrophils [12]. Consistent with this

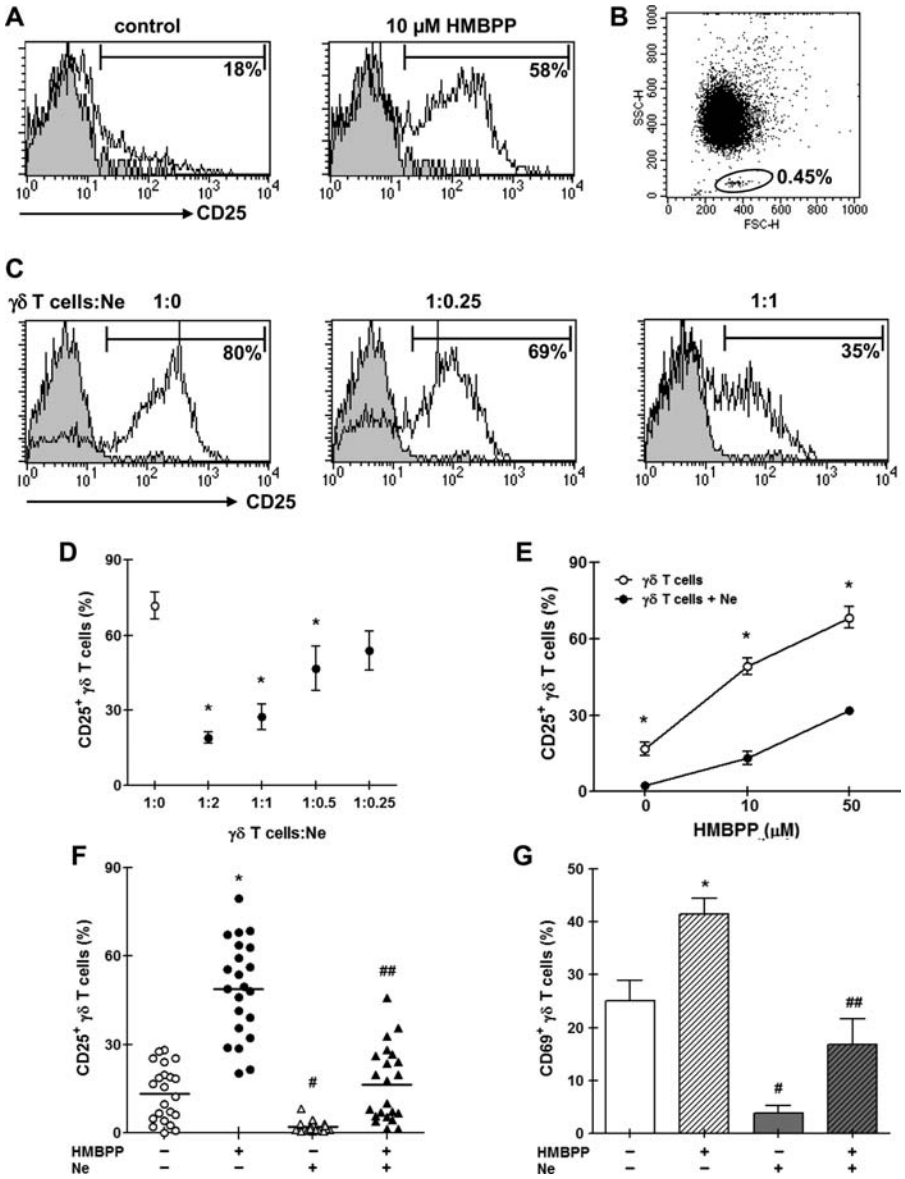

Figure 1. Neutrophils prevent the activation of γδ T cells. (A) γδ T cells were incubated with or without (control) HMBPP (10 μM) for 90 min at 37°C, washed, and cultured for 24 h. Then, the expression of CD25 by γδ T cells was analyzed by flow cytometry. Histograms from a representative experiment ($n = 22$) are shown. Markers indicate the percentage of cells expressing CD25. Gray histograms: isotype control. (B) A representative dot plot of purified neutrophils. The gate shows contaminating PBMCs (0.45%). (C, D) γδ T cells were incubated with HMBPP (10 μM) for 90 min at 37°C, washed, and cultured for 24 h in the absence or presence of neutrophils at different cell-to-cell ratios. (C) Histograms from a representative experiment out of $n = 4$ performed are shown. Gray histograms: isotype controls. (D) Data are shown as mean ± SEM of four independent experiments performed. $^{*}p < 0.05$ versus stimulated γδ T cells cultured without neutrophils, Wilcoxon matched ranked two-tailed test. (E) γδ T cells were incubated in the absence or presence of HMBPP (10 μM or 50 μM) for 90 min at 37°C, washed, and cultured for 24 h in the absence or presence of neutrophils, at a cell-to-cell ratio of 1:1. The expression of CD25 by γδ T cells was analyzed by flow cytometry. Data are shown as mean ± SEM of four independent experiments performed. $^{*}p < 0.05$ versus γδ T cells cultured with neutrophils, Wilcoxon matched ranked two-tailed test. (F, G) γδ T cells were incubated with HMBPP (10 μM) for 90 min at 37°C, washed, and cultured in the absence or presence of neutrophils, at a cell-to-cell ratio of 1:1 for 24 h. The expression of (F) CD25 ($n = 22$) and (G) CD69 on γδ T cells was then analyzed by flow cytometry. Data are shown as mean + SEM of $n = 5$ (G) experiments performed. * and $^{\#}p < 0.05$ versus unstimulated γδ T cells cultured alone, $^{\#\#}p < 0.05$ versus HMBPP-stimulated γδ T cells cultured without neutrophil.

evidence, we found that γδ T cells induced both the upregulation of CD11b and the release of myeloperoxidase to the extracellular medium (Fig. 4A–C). We then analyzed whether the activation of neutrophils by conventional agonists might further improve their ability to inhibit γδ T cells. To this aim, HMBPP-stimulated γδ T cells were cultured with neutrophils at different γδ T-cell: neutrophil ratios, in the absence or presence of the chemotactic peptide fMLP (1 μM). In parallel experiments, we observed that, as expected, 1 μM of fMLP induced the activation of neutrophils cultured alone measured as the enhancement in the expression of CD11b and the activation of the respiratory burst (data not shown). Figure 4D shows that activation by fMLP does not increase the ability of neutrophils to inhibit the upregulation of CD25 expression in γδ T cells.

We speculated that soluble products released by activated neutrophils might be responsible for the inhibition of γδ T-cell activation. To test this hypothesis, a new set of experiments were performed using 96-transwell chambers with a polycarbonate filter (0.4 μm pore size). Activated γδ T cells were included in the lower chamber of the transwell system while neutrophils were seeded in the upper chamber at a γδ T-cell:neutrophil ratio of 1:1 and 1:3. Controls were performed by incubating together γδ T cells and neutrophils at a cell-to-cell ratio of 1:1. Neutrophils prevented the upregulation of CD25 expression by γδ T cells even when both cell types were placed in different chambers of the transwell system indicating that suppression involves the participation of soluble factors released by neutrophils (Fig. 4E). However, it should be noted that a high neutrophil:γδ T-cell ratio (3:1) was necessary to obtain a significant inhibition under these experimental conditions suggesting that cell-to-cell contact might improve the ability of neutrophils to prevent the activation of γδ T cells. Interestingly, microscopic studies showed that neutrophils actually interact with γδ T cells (Fig. 4F). A quantitative analysis revealed that when cultured together at a γδ T cell:neutrophil ratio of 1:1 for 90 min at

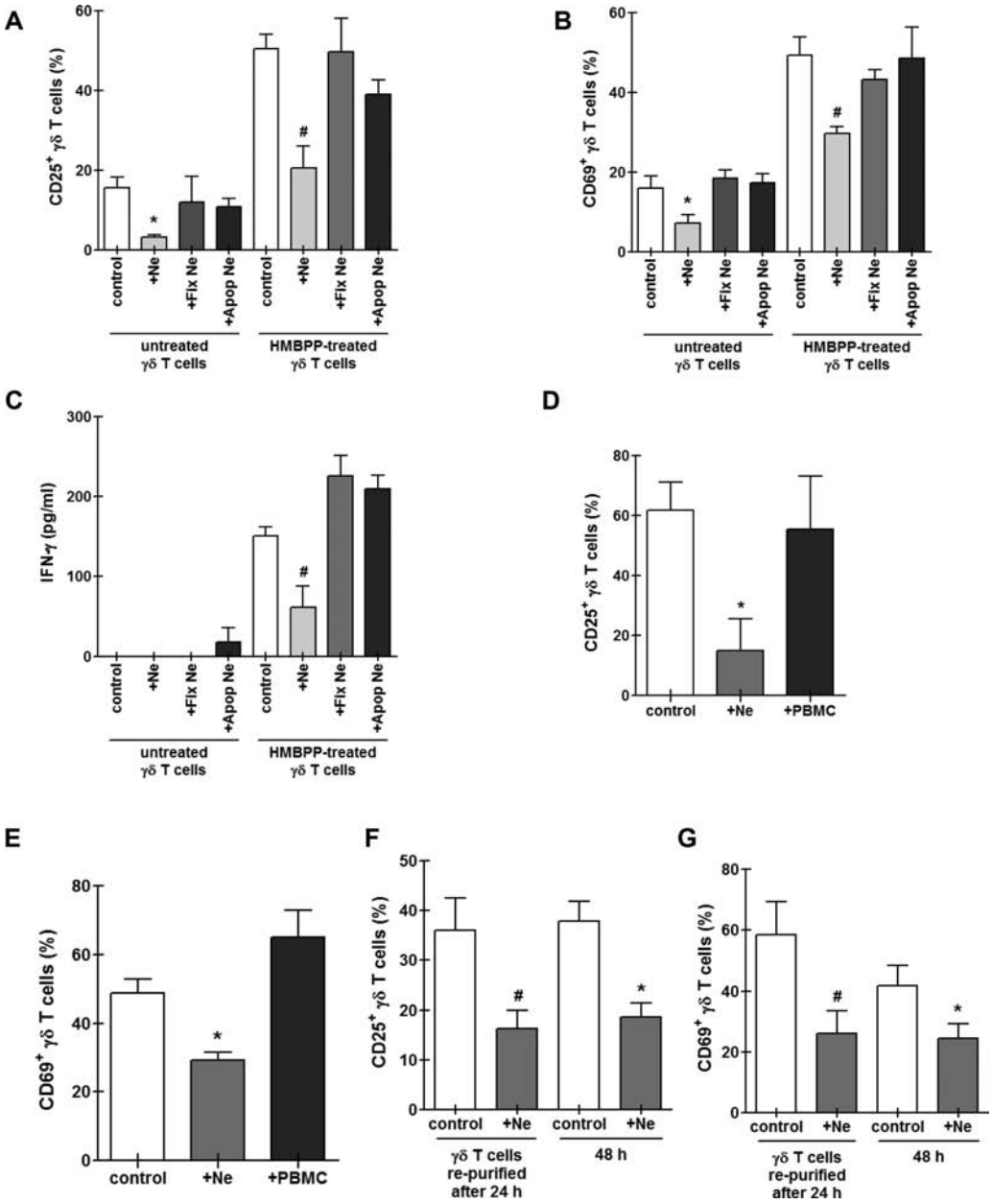

Figure 2. Neutrophil viability is required to prevent the activation of γδ T cells. (A–C) γδ T cells were cultured in the absence (untreated γδ T cells) or presence of HMBPP (10 μM) for 90 min at 37°C, washed, and cultured for an additional period of 24 h in the absence (control) or presence of freshly isolated autologous neutrophils (+Ne), apoptotic neutrophils (+Apop Ne) (>80% of apoptotic cells) or fixed neutrophils (+Fix Ne), using a cell-to-cell ratio of 1:1. Then, the expression of (A) CD25 and (B) CD69 on γδ T cells was analyzed by flow cytometry, and (C) the production of IFN-γ was evaluated in cell supernatants by ELISA. Data are shown as mean + SEM of four independent experiments, $^{*}p < 0.05$ versus untreated γδ T cells cultured alone, $^{\#}p < 0.05$ versus HMBPP-treated γδ T cells cultured alone, Friedman test for multiple comparisons with Dunn's posttest. (D, E) γδ T cells were incubated with HMBPP (10 μM) for 90 min at 37°C, washed, and cultured for an additional period of 24 h in the absence (control) or presence of freshly isolated neutrophils (+Ne) or PBMCs previously depleted of γδ T cells (+PBMC) at a cell-to-cell ratio of 1:1. Then, the expression of (D) CD25 and (E) CD69 on γδ T cells was analyzed by flow cytometry. Data are shown as mean + SEM of four independent experiments. $^{*}p < 0.05$ versus γδ T cells cultured alone, Mann–Whitney one-tailed test. (F, G) γδ T cells were incubated with HMBPP (10 μM) for 90 min at 37°C, washed, and cultured in the absence (controls) or presence of neutrophils (+Ne) at a cell-to-cell ratio of 1:1. After 24 h of co-culture, cells were recovered and γδ T cells were isolated. Purified γδ T cells were cultured for an additional period of 24 h in the absence of neutrophils. Another group of cells was cultured for 48 h in the absence (controls) or presence of neutrophils (+Ne) (cell-to-cell ratio of 1:1). The expression of (F) CD25 and (G) CD69 on γδ T cells was analyzed by flow cytometry. Data are shown as mean + SEM of four independent experiments. $^{\#}p < 0.05$ versus γδ T cells cultured alone during 24 h, repurified and cultured for an additional period of 24 h.$^{*}p < 0.05$ versus γδ T cells cultured alone during 48 h. Statistical significance determined by Kruskal–Wallis test for multiple comparisons with Dunn's posttest.

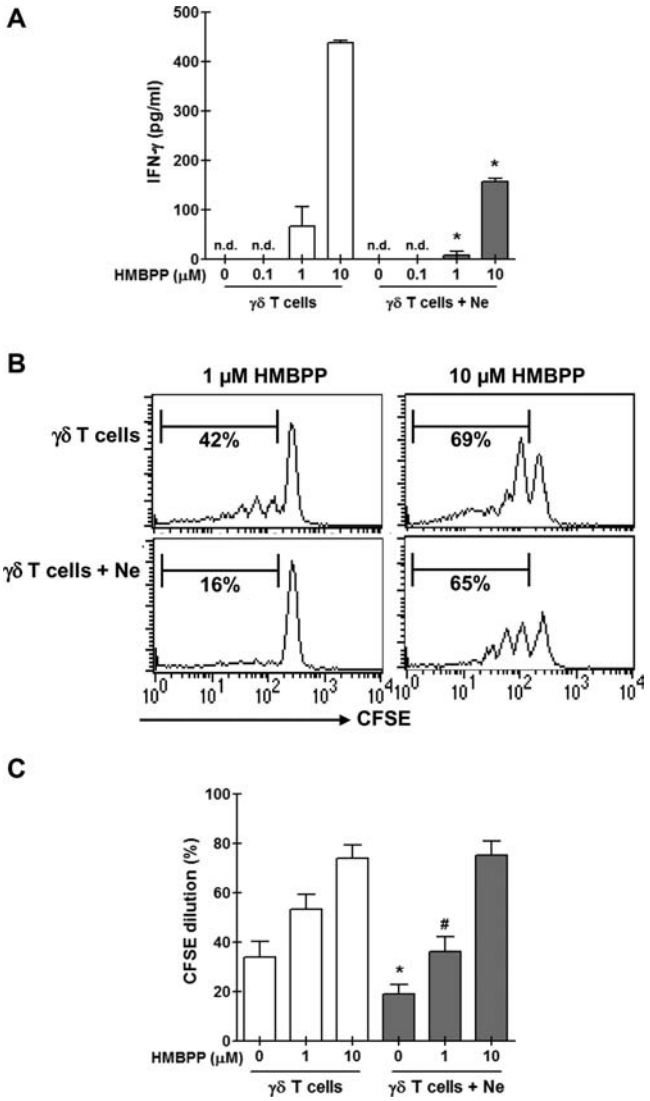

Figure 3. Neutrophils inhibit the production of IFN-γ and the proliferative response mediated by activated γδ T cells. (A) γδ T cells were incubated in the absence or presence of HMBPP (0.1, 1, and 10 μM) for 90 min at 37°C, washed, and cultured for an additional period of 24 h with or without neutrophils at a cell-to-cell ratio of 1:1. Then, the production of IFN-γ was analyzed in cell supernatants by ELISA. Data are shown as mean + SEM of five independent experiments. $^{*}p < 0.05$ versus activated γδ T cells cultured alone, Mann–Whitney two-tailed test. n.d.: nondetected. (B, C) γδ T cells were labeled with CFSE (5 μM, 20 min at 37°C) and incubated without (0) or with HMBPP (1 and 10 μM) for 90 min at 37°C. Cells were washed and cultured for 4 days in the absence (open bars) or presence (gray bars) of neutrophils at a cell-to-cell ratio of 1:1 in culture medium supplemented with human recombinant IL-2 (100 U/mL) (C). (B) The proliferation of γδ T cells was analyzed by flow cytometry. Histograms from a representative experiment of $n = 7$ performed are shown. Markers indicate the percentages of proliferation. (C) Data are shown as mean + SEM of seven independent experiments. $^{*}p < 0.05$ versus unstimulated γδ T cells cultured alone, $^{\#}p < 0.05$ versus γδ T cells stimulated with 1 μM of HMBPP and cultured without neutrophil, Mann–Whitney one-tailed test.

37°C, 24 ± 5% of the neutrophils form cell conjugates with γδ T cells (mean ± SEM, $n = 4$).

In a recent study, Pillay et al. [26] reported that a subset of neutrophils ($CD11c^{bright}/CD62L^{dim}/CD11b^{bright}/CD16^{bright}$) suppresses the proliferation of conventional αβ T cells through a cell-to-cell contact-dependent mechanism, which requires the participation of the neutrophil integrin Mac-1 (CD11b/CD18). To reveal whether Mac-1 might also be involved in the inhibition of γδ T cells mediated by neutrophils, we used a blocking mAb directed to CD18 (clone TS1/18), previously tested by its ability to prevent the adhesion of activated neutrophils to fibrinogen-coated plates (data not shown). Results in Fig. 4G–I show that blocking of CD18 did not affect the ability of neutrophils to suppress the activation of γδ T cells measured as the upregulation of CD25 and CD69 expression and the stimulation of IFN-γ production.

Different products released by activated neutrophils might be able to modulate the function of γδ T cells. We analyzed a possible role for prostaglandin E2 (PGE_2), arginase I, and ROS. As previously described [27, 28], PGE_2 markedly prevented the activation of γδ T cells (Fig. 5A and B). However, indomethacin, at concentrations able to completely abrogate the aggregation of platelets induced by arachidonic acid (insert in Fig. 5C), which depends on the production of PGE_2 by the platelets themselves [29], did not disturb the ability of neutrophils to prevent the upregulation of CD25 in activated γδ T cells (Fig. 5C). The role of arginase I was analyzed by using the arginase inhibitors *N*-hydroxy-L-arginine (NOHA) [30] and L-valine, and also by adding L-arginine to the culture medium [31]. No effect was induced by these compounds (Fig. 5D–F).

To analyze a possible role for ROS, we first determined whether γδ T cells were actually able to induce the production of ROS in neutrophils. To this aim, we analyzed the production of hydrogen peroxide by flow cytometry and confocal microscopy using dihydrorhodamine 123 (DHR), a dye which emits fluorescence upon oxidation by hydrogen peroxide. Results in Figure 6A and B show that γδ T cells effectively induced the production of hydrogen peroxide by neutrophils. More importantly, Figure 6C and D shows that catalase significantly impaired the ability of neutrophils to prevent the upregulation of CD25 and CD69 expression in stimulated γδ T cells, suggesting the participation of hydrogen peroxide. The fact that the ROS-generating system xanthine/xanthine oxidase inhibited the upregulation of CD25 and CD69 expression in activated γδ T cells in a similar fashion than neutrophils (Fig. 6E and F), without affecting cell viability (data not shown), also suggested that neutrophil-derived ROS are involved in the suppression of γδ T cells. This was confirmed by using neutrophils isolated from patients with chronic granulomatous disease (CGD). They exerted a weak inhibitory effect on the upregulation of CD25 expression and completely failed to inhibit the production of IFN-γ by activated γδ T cells (Fig. 6G and H). Finally, we wonder about the mechanisms through which γδ T cells stimulate the production of ROS by neutrophils. Since activated Vγ9Vδ2 T cells secrete large amounts of IFN-γ and TNF-α [32], we analyzed the role of these cytokines by using blocking antibodies directed to IFN-γ and

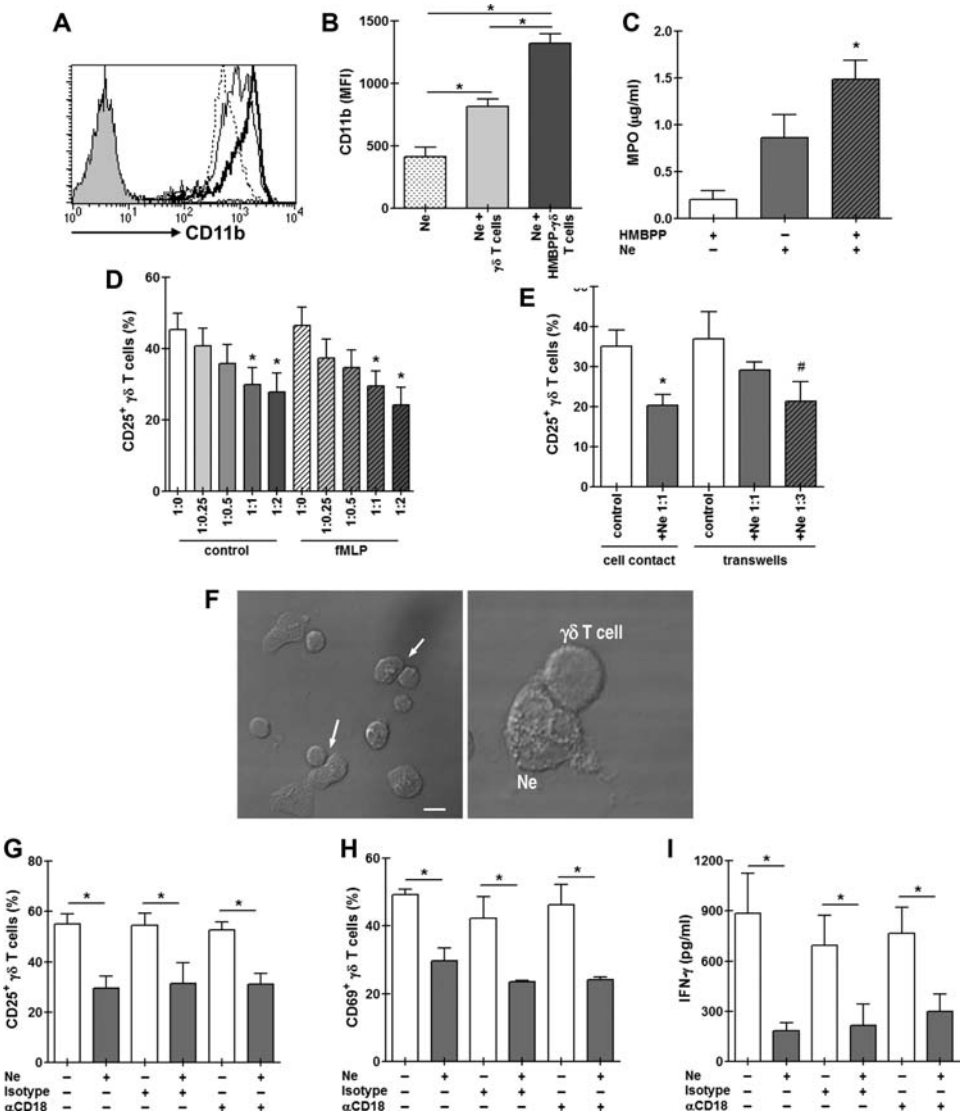

Figure 4. Soluble factors released by neutrophils inhibit the activation of γδ T cells. (A–C) γδ T cells were incubated in the absence or presence of HMBPP (10 μM) for 90 min at 37°C, washed, and cultured for an additional period of 3 h with neutrophils at a cell-to-cell ratio of 1:1. (A, B) The expression of CD11b by neutrophils was analyzed by flow cytometry and (C) the release of myeloperoxidase (MPO) was quantified in cell supernatants using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. (A) Histograms from a representative experiment of four independent experiments performed are shown. Gray histogram: isotype control; dotted line histogram: neutrophils cultured alone; thin line histogram: neutrophils incubated with nonstimulated γδ T cells; and thick line histogram: neutrophils incubated with HMBPP-stimulated γδ T cells. (B) Data are also shown as mean + SEM of four independent experiments performed, $^{*}p < 0.05$, Mann–Whitney two-tailed test. (C) Data are shown as mean + SEM of four independent experiments performed. $^{*}p < 0.05$ versus neutrophils cultured with unstimulated γδ T cells, Friedman test for multiple comparisons with Dunn's posttest. (D) γδ T cells were incubated with HMBPP (10 μM) for 90 min at 37°C, washed, and cultured for 24 h in the absence (1:0) or presence of neutrophils at different cell ratios (γδ T cell:Ne ratio of 1:0.25, 1:1, and 1:2), without (control) or with fMLP (1 μM). The expression of CD25 by γδ T cells was analyzed by flow cytometry and the percentage of CD25-positive cells shown as mean + SEM of seven independent experiments performed.$^{*}p < 0.05$ versus activated γδ T cells cultured alone, Kruskal–Wallis test for multiple comparisons with Dunn's posttest. (E) γδ T cells were incubated with HMBPP (10 μM) for 90 min at 37°C, washed, and seeded in the lower compartment of a 0.4 μm pore size membrane transwell system and cultured in the absence (control) or presence of neutrophils. Neutrophils were seeded in the upper chamber at a γδ T-cell:neutrophil ratios of 1:1 (+Ne 1:1) and 1:3 (+Ne 1:3). Controls were performed by incubating together γδ T cells and neutrophils at a cell-to-cell ratio of 1:1. Cells were cultured for 24 h and the expression of CD25 by γδ T cells was then analyzed by flow cytometry. Data are shown as mean + SEM of four independent experiments performed. $^{*}p < 0.05$ versus activated γδ T cells cultured alone $^{\#}p < 0.05$ versus activated γδ T cells cultured alone, Friedman test for multiple comparisons with Dunn's posttest. (F) γδ T cells were incubated with HMBPP (10 μM) for 90 min at 37°C, washed and incubated with neutrophils during 90 min at 37°C at a cell-to-cell ratio of 1:1. Cells were fixed and the formation of cell conjugates was analyzed by microscopy. Representative images are shown. Arrows indicate the presence of conjugates. Right panel shows a magnification of a cell conjugate. Bar: 10 μm. (G–I) γδ T cells were incubated with HMBPP (10 μM) for 90 min at 37°C, washed and treated with saturating concentrations (30 μg/mL) of a blocking mAb directed to CD18 (αCD18) or with an isotype control (30 μg/mL) during 30 min. Then, cells were cultured for an additional period of 24 h in the absence (open bars) or presence (gray bars) of neutrophils at a cell-to-cell ratio of 1:1. The expression of (G) CD25 ($n = 10$) and (H) CD69 ($n = 5$) was then analyzed by flow cytometry and shown as mean + SEM of $n = 10$ (G) and $n = 5$ (H) experiments performed. (I) The production of IFN-γ was evaluated in cell supernatants by ELISA and shown as mean + SEM of five experiments performed, $^{*}p < 0.05$, Kruskal–Wallis test for multiple comparisons with Dunn's posttest.

the TNF-α blocker etanercept. Treatment with etanercept, but not with blocking antibodies directed to IFN-γ, abrogated the production of ROS by neutrophils as well as their ability to suppress γδ T cells (Fig. 6I and J). However, etanercept also inhibited the activation of γδ T cells (Fig. 6J), suggesting that TNF-α is involved not only in the stimulation of ROS production by neutrophils but also in the proper activation of γδ T cells.

Discussion

Neutrophils are the most abundant circulating leukocyte in the human and play a major role in the immune response against bacteria and fungi infection [17]. They ingest and kill microbes through a large variety of antimicrobial weapons that include ROS, proteolytic enzymes, antimicrobial peptides, and extracellular traps [16, 17, 33]. Neutrophils have long been viewed as terminally differentiated and short-lived effector cells devoid of functional plasticity [34]. In the past few years, however, this limited view has been challenged and it is now clear that the function of neutrophils cannot be merely explained in terms of phagocytosis, killing, and degradation of internalized pathogens [34, 35]. In fact, neutrophils produce a broad array of cytokines and chemokines [36], modulate the function of immune cells such as macrophages, dendritic cells, NK cells, and conventional T cells, and are also able to acquire different functional profiles [37–41], a matter that has been the subject of many studies in the field of antitumor immunity [42].

Here, we show for the first time that freshly isolated neutrophils prevent the activation of γδT cells. Neutrophils efficiently inhibited the upregulation of CD25, CD69, and the production of IFN-γ by γδ T cells stimulated by HMBPP. The expansion of γδ T cells was also inhibited by neutrophils, however, the inhibitory effect was observed using 1 μM but not 10 μM of HMBPP. We do not actually know why neutrophils inhibit γδ T-cell proliferation at 1 μM but not 10 μM HMBPP. It should be noted, however, that the other responses analyzed (upregulation of CD25 and CD69 expression and IFN-γ production) were evaluated at 24–48 h after stimulation while cell proliferation was analyzed after 4 days of culture. We speculate that this long period might enable γδ T cells to overcome (at least to some degree) the inhibitory action exerted by neutrophils especially when high concentrations of HMBPP were used as stimulus. Further experiments, however, are required to test this hypothesis.

Suppression of γδ T-cell function required the viability of neutrophils, in fact, either apoptotic or fixed neutrophils failed to prevent the activation of γδ T cells. Additional experiments performed in transwell chambers revealed that the inhibition of γδ T cells by neutrophils was also observed when both cell types were separated by a transwell filter indicating that it involves the participation of soluble factors. However, it should be noted that under these experimental conditions a high neutrophil:γδ T-cell ratio was required to achieve a significant inhibitory effect, suggesting that although the physical interaction between both cell types does not represent an absolute requirement, it might improve the ability of neutrophils to prevent the activation of γδ T cells.

Previous studies have demonstrated that human neutrophils are able to inhibit the activation of conventional T cells [26]. αβ T-cell suppression mediated by neutrophils appears to involve the participation of the L-arginine metabolizing enzyme arginase I as well as the production of ROS [26, 43, 44]. Munder et al. [43] have reported that arginase released by death neutrophils induces the depletion of arginine in the αβ T-cell environment, leading to CD3ζ chain downregulation, which compromises efficient T-cell signaling without affecting T-cell viability. The inhibitory effect was transient and T cells resumed their potential to proliferate and secrete cytokines when they were stimulated again in an arginine-supplemented environment [43]. Rotondo et al. [44] have recently confirmed the ability of arginase I to inhibit the activation of conventional T cells. On the other hand, Pillay et al. [26] identified a subset of human mature neutrophils able to suppress T-cell proliferation during the course of an acute systemic inflammation induced by endotoxin challenge. The inhibition of αβ T-cell response was related to the local release of hydrogen peroxide from the neutrophils into the immunological synapse between neutrophils and T cells, which involves the participation of the neutrophil integrin Mac-1 [26]. Interestingly, not only the function of conventional T cells but also the activity of invariant NKT cells appears to be under the control of neutrophils [45]. Wingender et al. [45] have recently shown that neutrophils inhibit the function of invariant NKT-cell function in mice and humans, both under resting conditions and during inflammation, through a cell-to-cell contact-dependent mechanism that has not been yet characterized.

Neither arginase I nor PGE_2 appears to be involved in the inhibition of γδ T cells mediated by neutrophils. Our results suggest that γδ T cells effectively induce the production of ROS by neutrophils, which in turn suppress γδ T cells. In fact, we found that catalase significantly prevented the suppression of γδ T cells while the ROS-generating system xanthine/xanthine oxidase reproduced the inhibitory effect mediated by neutrophils. Moreover, neutrophils from CGD patients exerted a weak inhibitory effect on the upregulation of CD25 expression and completely failed to suppress the production of IFN-γ by γδ T cells. Interestingly, we found that the TNF-α blocker etanercept abrogated the ability of γδ T cells to stimulate the production of ROS by neutrophils as well as the ability of neutrophils to suppress γδ T cells, suggesting an important role for TNF-α in the cross-talk between both cell populations. It should be noted, however, that etanercept also inhibited the activation of γδ T cells by HMBPP indicating that TNF-α is involved in the activation of γδ T cells induced by phosphoantigens. This observation is consistent with previous results published by Ueta and coworkers showing that the activation of γδ T cells induced by IL-12 was inhibited by blocking antibodies directed to TNF-α [46]. These results suggest a central role for the autocrine production of TNF-α in the activation of γδ T cells triggered by different stimuli.

Interestingly, some studies have reported the expansion of γδ T cells in neutropenic patients [47–49]. These observations might indicate that neutrophils are able to control the pool size of γδ

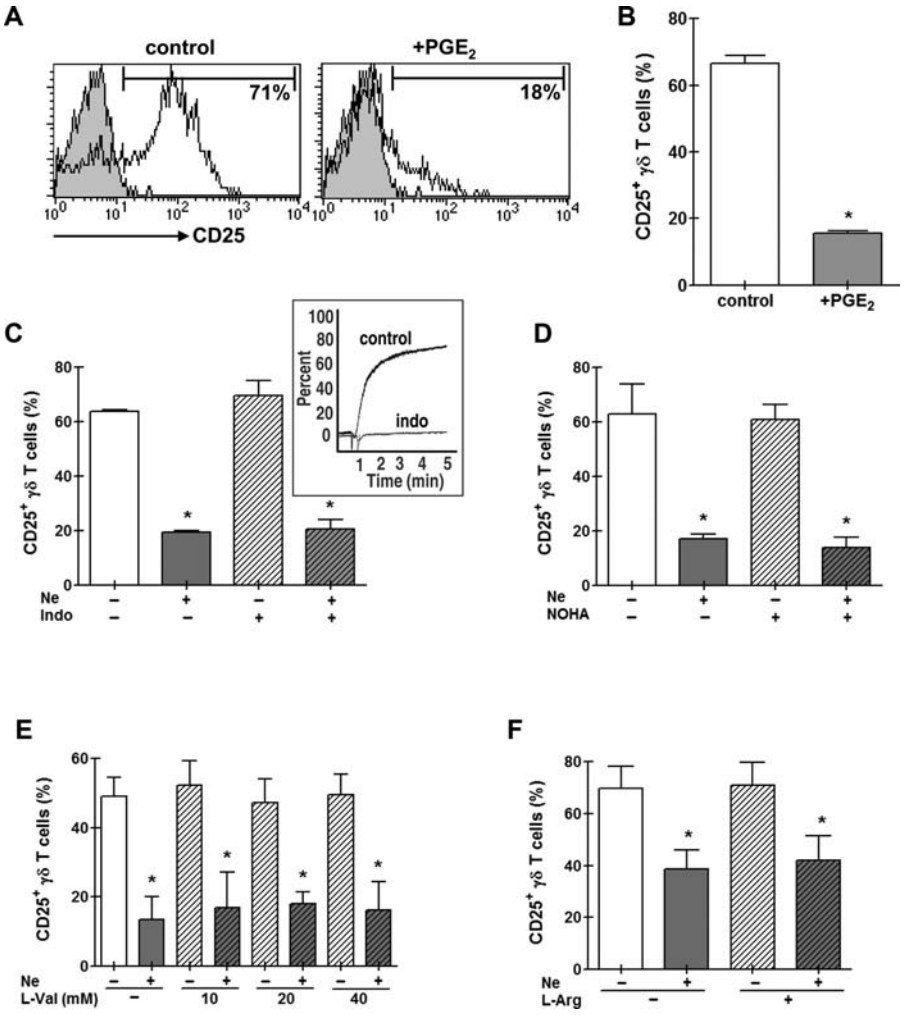

Figure 5. Analysis of the mechanisms through which neutrophils prevent the activation of γδ T cells. (A–C) γδ T cells were incubated with HMBPP (10 μM) for 90 min at 37°C, washed, and cultured (A, B) for an additional period of 24 h without (control) or with PGE_2 (2 μg/mL) or (C) in the absence or presence of neutrophils treated with indomethacin (10 μM) (Indo). The expression of CD25 was analyzed by flow cytometry. (A) Histograms from a representative experiment out of three independent experiments performed are shown. Markers represent the percentage of CD25 expression. Gray histogram: isotype control. (B) Data are shown as mean + SEM of three experiments performed, $^*p < 0.05$ versus activated γδ T cells cultured without PGE_2 (control), Wilcoxon matched ranked one-tailed test. (C) The inset figure shows the abrogation of platelet aggregation triggered by arachidonic acid by indomethacin 10 μM. (D–F) γδ T cells were incubated with HMBPP (10 μM) for 90 min at 37°C, washed, and cultured for an additional period of 24 h with neutrophils at a cell-to-cell ratio of 1:1 (D) in the absence or presence of N-hydroxy-L-arginine (400 μm), (E) L-Valine (L-Val) or (F) L-Arginine (L-Arg, 5 mM). The expression of CD25 by γδ T cells was analyzed by flow cytometry. Data are shown as the mean + SEM of four independent experiments performed. $^*p < 0.05$ versus activated γδ T cells cultured alone, Kruskal–Wallis test for multiple comparisons with Dunn's posttest.

T cells, a presumption that requires further confirmation. In summary, our results suggest a novel regulatory axis whereby neutrophils suppress the function of γδ T cells. While γδ T cells stimulate the recruitment and the activation of neutrophils during the course of infectious processes, neutrophils in turn might suppress the activation of γδ T cells in order to contribute to the resolution of inflammation once the infection has been resolved.

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, FBS, CFSE, and DHR were from Invitrogen (Carlsbad, CA, USA). PE-conjugated mouse antibodies directed to CD11b and CD25, PerCP-conjugated mouse antibody directed to CD69, isotype controls, and recombinant human IL-2 were from BD Bioscience (San Jose, CA, USA). HMBPP was obtained from Cayman Chemical (Ann Arbor, MI, USA). Human IFN-γ ELISA kit was from BD Biosciences (San Diego, CA, USA). Fluoromount G, PGE_2, indomethacin, L-valine, L-arginine, PMA, and xanthine were obtained from Sigma-Aldrich (Saint Louis, MO, USA). N-hydroxy-L-arginine was kindly provided by Dr. Sales from CEFYBO-CONICET, School of Medicine, Buenos Aires University (Argentina). Catalase was from Worthington Biochemical Corporation (Lakewood, NJ, USA), anti-CD18 blocking mAb (TS1/18) was from American Type Culture Collection (Manassas, VA, USA), anti-IFN-γ blocking mAb was from BioLegend (San Diego, CA), xanthine oxidase was from Roche (Germany), and etanercept was from Wyeth, Buenos Aires,Argentina.

γδ T-cell purification and culture

Peripheral blood samples were obtained from healthy donors, volunteers, or CGD patients, after institutional Ethical Committee approval. Donors provided written informed consent before the collection of the samples. PBMCs 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

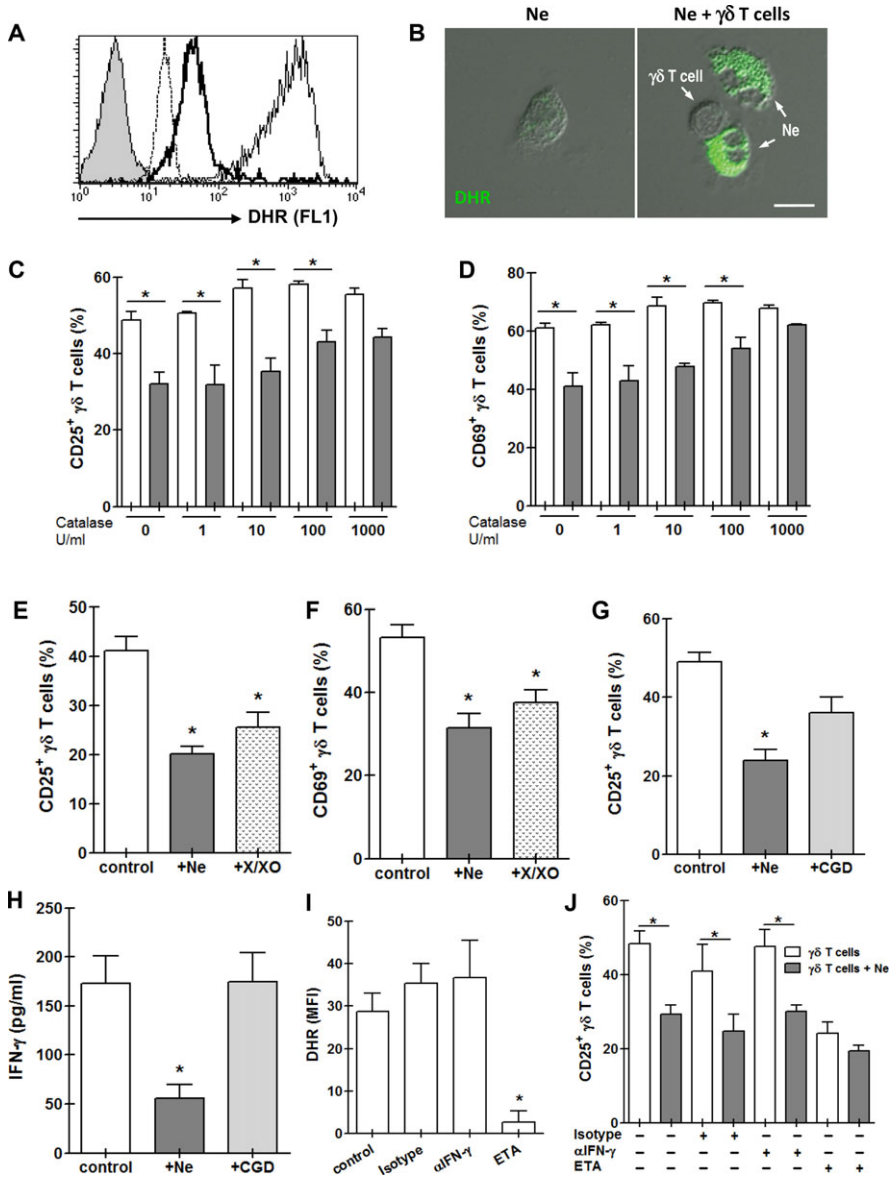

Figure 6. Neutrophils inhibit γδ T-cell activation through a ROS-dependent mechanism. (A, B) Neutrophils loaded with DHR were incubated alone, with HMBPP-activated γδ T cells (cell-to-cell ratio of 1:1) or with PMA (100 ng/mL) during 20 min at 37°C and then analyzed by (A) flow cytometry and (B) fluorescence microscopy. (A) Histogram is from a single experiment representative of three performed. Gray histogram: neutrophils nonloaded with DHR; dotted line histogram: neutrophils loaded with DHR cultured alone; thick line histogram: neutrophils loaded with DHR and incubated with HMBPP-stimulated γδ T cells; and thin line histogram: neutrophils loaded with DHR and activated by PMA. (B) Images shown are from one experiment representative of three experiments performed. Bar: 10 μm. (C, D) γδ T cells were incubated with HMBPP (10 μM) for 90 min at 37°C, washed and cultured for an additional period of 24 h with neutrophils at a cell-to-cell ratio of 1:1, in the absence or presence of different concentrations of catalase. The expression of (C) CD25 and (D) CD69 by γδ T cells was analyzed by flow cytometry. Data are shown as mean + SEM of four independent experiments performed. $^*p < 0.05$, Kruskal–Wallis test for multiple comparisons with Dunn's posttest. (E, F) γδ T cells were stimulated with HMBPP (10 μM) for 90 min at 37°C, washed, and cultured for an additional period of 24 h with neutrophils (cell-to-cell ratio of 1:1) or with xanthine (100 μM) and xanthine oxidase (1 mU/mL) (X/XO). Then, the expression of (E) CD25 and (F) CD69 by γδ T cells was analyzed by flow cytometry. Data are shown as mean + SEM of six independent experiments performed. $^*p < 0.05$ versus activated γδ T cells cultured alone, Kruskal–Wallis test for multiple comparisons with Dunn's posttest. (G, H) γδ T cells were isolated from healthy donors and stimulated with HMBPP (10 μM). Then, cells were cultured in the absence (control) or presence of allogeneic neutrophils obtained from healthy donors (Ne) or chronic granulomatous disease (CGD) patients using a cell-to-cell ratio of 1:1. After 24 h, (G) the expression of CD25 was analyzed by flow cytometry and (H) the production of IFN-γ in cell supernatants was evaluated by ELISA. Data are shown as mean + SEM of four experiments performed, $^*p < 0.05$ versus γδ T cells cultured alone, Kruskal–Wallis test for multiple comparisons with Dunn's posttest. (I, J) γδ T cells were stimulated with HMBPP (10 μM) for 90 min at 37°C, washed, and treated or not (control) with a blocking mAb directed to IFN-γ (10 μg/mL) (αIFN-γ), an isotype control (10 μg/mL), or etanercept (ETA, 25 μg/mL), during 30 min at 37°C. (I) Cells were then incubated with DHR-loaded neutrophils at a cell-to-cell ratio of 1:1, and after 20 min of incubation at 37°C the population of neutrophils was analyzed by flow cytometry. Data are expressed as the MFI of DHR and shown as mean + SEM of four independent experiments. $^*p < 0.05$ versus control, Mann–Whitney one-tailed test. (J) γδ T cells were cultured for an additional period of 24 h with neutrophils at a cell-to-cell ratio of 1:1. Then, the expression of CD25 by γδ T cells was analyzed by flow cytometry and shown as mean + SEM of four independent experiments. $^*p < 0.05$, Mann–Whitney one-tailed test.

MicroBead isolation kit, according to the manufacture's recommendations. The purity of recovered cells was higher than 95% in all the experiments as measured by flow cytometry. Cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS.

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. The purity was checked by flow cytometry (>98%). Unless otherwise stated, co-cultures of γδ T cells and neutrophils were made using autologous cells. For neutrophil fixation, cells were incubated with 3% paraformaldehyde during 20 min, then cells were washed and suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS.

Immunostaining and flow cytometry

γδ T cells were stained with PE-conjugated antibodies directed to CD25 and PerCP-conjugated antibodies against CD69. 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 FACS flow cytometer and CellQuest software (BD Biosciences).

Detection of IFN-γ by ELISA

γδ T cells (1×10^6/mL) were incubated in the absence or presence of HMBPP (0.1–10 μM, 90 min at 37°C). Then, cells were washed and 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 was analyzed by ELISA, according to the manufacturer's recommendations.

Proliferation assay

γδ T cells were labeled with 5 μM CFSE for 20 min at 37°C and then washed three times with culture medium. Afterward, cells were pretreated, or not, with HMBPP (1 and 10 μM, 90 min at 37°C), washed and then transferred to a 96-well culture plate with or without autologous neutrophils. After 4 days of culture with human recombinant IL-2 (100 U/mL), cells were collected and CFSE dilution was evaluated in γδ T cells by flow cytometry.

Transwell co-culture

γδ T cells (1×10^6/mL) were stimulated with HMBPP (10 μM, 90 min at 37°C), washed and seeded in the lower chamber of a 96-transwell chamber with a polycarbonate filter of 0.4 μm pore size (Corning, MA, USA). Afterward, autologous neutrophils were added in the upper chamber, at γδ T cell:neutrophil ratios of 1:1 and 1:3. Controls were performed by incubating together γδ T cells and neutrophils at a cell-to-cell ratio of 1:1. After 24 h of culture at 37°C, γδ T cells were recovered, stained for CD25, and analyzed by flow cytometry.

Analysis of cell conjugates

γδ T cells stimulated with HMBPP (10 μM, 90 min) were immobilized on poly-L-lysine-coated glass cover slips (12 mm) during 20 min at room temperature. The cover slips were washed, and culture medium was added to further incubate the attached cells at 37°C and 5% CO_2 for 30 min. Then, autologous neutrophils were added on each cover slip and incubated for 90 min at 37°C. After incubation, the cover slips were carefully washed with PBS and cells were fixed in 3% paraformaldehyde. Cover slips were then 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 and images were analyzed using the Olympus FV10-ASW software.

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 autologous γδ T cells previously stimulated with HMBPP (10 μM, 90min) at a cell-to-cell ratio of 1:1. As a control, neutrophils were stimulated with PMA (100 ng/mL). After 20 min at 37°C, cells were analyzed by flow cytometry and confocal microscopy. For flow cytometry analysis, the variation of the MFI emission of DHR was determined in the gate of neutrophils. For microscopy studies, cells were mounted onto glass slides using Fluoromount-G solution. Immunofluorescence images were acquired with a FluoView FV1000 confocal microscope (Olympus, Tokio, Japan) as described above.

Statistical analysis

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

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Abbreviations: **CGD**: chronic granulomatous disease · **DHR**: dihydrorhodamine · **HMBPP**: (E)-1-hydroxy-2-methylbut-2-enyl 4-diphosphate · **PGE2**: prostaglandin E_2

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