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Hypertonic saline increases $\gamma\delta$ T cell-mediated killing of activated neutrophils

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Abstract

Objective—Hypertonic saline fluids used to resuscitate trauma patients can prevent neutrophil-mediated lung tissue damage, making them attractive alternatives to conventional resuscitation fluids. We have previously shown that $\gamma\delta$ T cells, a small T lymphocyte subset, reduce acute inflammatory lung damage by eliminating activated neutrophils that express heat shock protein 72 on the cell surface. Here, we studied whether these protective effects of hypertonic saline are related to improved $\gamma\delta$ T cell-mediated neutrophil killing.

Design—Laboratory investigation.

Setting—University research laboratory.

Subjects—Human peripheral blood from healthy subjects—isolated $\gamma\delta$ T lymphocytes and neutrophils.

Interventions—Isolated blood cells were treated with different concentrations of hypertonic saline and endotoxin of *Escherichia coli* O111:B4 (lipopolysaccharide). In some experiments, $\gamma\delta$ T cells were activated by CD3 cross-linking or by phorbol-myristate acetate and ionomycin, or by phytohemagglutinin.

Measurements and Main Results—Clinically relevant concentrations of hypertonic saline (20 mM) significantly augmented CD69 expression of $\gamma\delta$ T cells that were stimulated with 100 ng/mL lipopolysaccharide. Additionally, lipopolysaccharide induced a three- to five-fold increase in tumor necrosis factor- α and interleukin-10 expression by $\gamma\delta$ T cells. This response was completely abrogated by hypertonic saline. These data indicate that hypertonic saline can modulate $\gamma\delta$ T cell functions. Stimulation of neutrophils with 1–1,000 ng/mL lipopolysaccharide caused a greater than 3-fold increase in heat shock protein-72 expression on the cell surface, which was significantly augmented by hypertonic saline. In cocultures of $\gamma\delta$ T cells with autologous neutrophils, $15.6 \pm 3.4\%$ of all neutrophils were killed within 120 min. In the presence of lipopolysaccharide (1 μ g/mL), this percentage increased to $23.7 \pm 2.1\%$, and it was further increased to $31.8 \pm 3.1\%$ when 20 mM hypertonic saline was added with lipopolysaccharide.

Conclusions—Our findings suggest that hypertonic saline enhances the elimination of inflammatory neutrophils by $\gamma\delta$ T cells by augmenting heat shock protein-72 expression on the cell surface of neutrophils. Hypertonic saline resuscitation may therefore protect host tissues by enhancing neutrophil clearance from the lungs.

Keywords

endotoxin; $\gamma\delta$ T cell receptor-positive lymphocytes; hypertonicity; cytotoxicity; immunomodulation

Hypertonic saline (HS) resuscitation effectively restores blood pressure in trauma patients with major blood loss (1). We have previously demonstrated that HS enhances the ability of T cells to proliferate and to synthesize key cytokines such as interleukin (IL)-2 *in vitro* and that HS resuscitation enhances T-cell function *in vivo*, promising to reduce the risks of posttraumatic sepsis (2, 3). Furthermore, we found that HS resuscitation reduces lung tissue damage after trauma and hemorrhage by limiting neutrophil-mediated lung tissue damage (4, 5). The mechanisms underlying these beneficial effects of HS resuscitation are not yet completely understood.

Lymphocytes carrying T-cell receptors (TCR) comprising of γ and δ subunits are referred to as $\gamma\delta$ T cells. The majority of this T-cell subpopulation is found not in blood but in tissues that are most susceptible to infections such as the skin, lungs, gut, and urinary tract. Although their function is still not well understood, it has become clear that $\gamma\delta$ T cells play a major role in the regulation of the host response to inflammatory challenges (6, 7). $\gamma\delta$ T cells carry out this function by producing regulatory cytokines such as tumor necrosis factor (TNF)- α and IL-10 and through direct cytotoxic effects on compromised host cells (6, 8, 9). $\gamma\delta$ T cells accumulate at sites of acute inflammation, where they control tissue destruction resulting from collateral damage inflicted by the host's phagocyte population that infiltrates inflamed tissues (10). Because of limited germ line-encoded diversity of the $\gamma\delta$ TCR, the response of $\gamma\delta$ T cells is generally committed to evolutionarily conserved antigen repertoires associated with pathogenic microorganisms (7, 11). For example, a large portion of $\gamma\delta$ T cells responds to peptides related to bacterial heat shock proteins (Hsp) (12). Hsp are ligands of the TCR of murine and human $\gamma\delta$ T cells, and when expressed on the surface of host cells, Hsp molecules are interpreted by $\gamma\delta$ T cells as pathogen-associated molecular patterns that trigger a cytotoxic immune response, which leads to the killing of the affected host cells (13, 14).

Recently, we demonstrated that activated neutrophils express Hsp72 on the cells surface and that $\gamma\delta$ T cells recognize these molecules, resulting in the rapid elimination of neutrophils from the lungs of septic mice (13). Expression of inducible Hsp72 on the outer cell membrane of neutrophils has also been reported in critically ill patients after major trauma (15). In these patients, neutrophils are responsible in large part for severe complications, such as acute inflammatory lung damage and multiple organ failure (16). Furthermore, the influx and the activation of neutrophils frequently coincide with the suppression of T cell function, resulting in immune disorders that further deteriorate the clinical course of

critically ill patients (17). Therapeutic approaches that target these processes may limit the severity of the clinical complication in such patients.

Although the enhancing effects of HS on conventional T cells have been firmly established, there is no information available on how HS affects the responses of the $\gamma\delta$ T cell subpopulation. In the present study, we examined how $\gamma\delta$ T cells respond to HS and whether HS modulates the ability of $\gamma\delta$ T cells to kill activated neutrophils.

MATERIALS AND METHODS

$\gamma\delta$ T Cell and Neutrophil Isolation

Peripheral blood samples from individual healthy adult volunteers ($n = 7$) were collected by venipuncture using standard heparinized Vacutainer tubes (Becton Dickinson and Co., Franklin Lakes, NJ). The entire study was completed with blood samples from this set of donors. The blood of the donors was not pooled for these studies; autologous cocultures of polymorphonuclear neutrophils (PMN) and $\gamma\delta$ T cells were performed with cells obtained from the same individuals. Informed consent was obtained from all participants, and all human experiments were approved by the Human Research Programs of the Beth Israel Deaconess Medical Center and the University of California San Diego. Peripheral blood mononuclear cells and PMN were isolated using a Percoll density gradient centrifugation described previously (13). An enriched fraction of $\gamma\delta$ T cells was prepared using a MACS system (Miltenyi Biotec Inc., Auburn, CA) as described previously (13). Allophycocyanin-labeled mouse anti-human $\gamma\delta$ TCR mAb (clone B1; BD Biosciences Pharmingen, San Jose, CA) and magnetic microbeads conjugated to anti-allophycocyanin IgG (Miltenyi Biotec) were used for positive selection of $\gamma\delta$ T cells from peripheral blood mononuclear cells. PMN and $\gamma\delta$ T cell preparations had a purity of $>96\%$ and 85% , respectively, as assessed by flow cytometry. Cell viability was $>90\%$ as determined by trypan blue and propidium iodide staining (PI; Sigma-Aldrich Corp, St. Louis, MO). Isolated cells were resuspended in RPMI-1640 cell culture medium (HyClone, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (Omega Scientific Inc., Tarzana, CA) and incubated at 37°C and $5\% \text{ CO}_2$ as described below. All experiments were performed in a total reaction volume of 0.2 mL . These assay mixtures were made hypertonic by adding HS at concentrations required to increase the culture tonicity by 20 , 40 , or 100 mM beyond isotonic levels. For that purpose, we used appropriate dilutions of culture medium containing an additional 1 M NaCl. The HS concentrations in the assay were chosen to encompass the hypertonicity levels achieved by hypertonic resuscitation of trauma patients.

Flow Cytometry

Cell samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Lincoln Park, NJ). $\gamma\delta$ T cells were identified by double-staining with anti- $\gamma\delta$ TCR and anti-CD3 mAb (clones B1 and UCHT1; BD Biosciences, San Jose, CA). After gating of lymphocytes using forward and side scatter properties, the percentage of double-positive cells was determined. PMN were identified and gated based on their forward and side scatter characteristics and PMN gating was verified by staining with mouse anti-human CD66b mAb (clone G10F5; BD Biosciences Pharmingen). For each assay $10,000$ cells were

collected. In parallel, autofluorescence controls and respective isotype controls (Sigma) were included in each analysis.

$\gamma\delta$ T Cell Activation

Isolated $\gamma\delta$ T cells were incubated for 20 hrs with or without the indicated concentrations of HS and lipopolysaccharide (LPS) (*Escherichia coli* O111:B4; Sigma). In some experiments, $\gamma\delta$ T cells were activated by crosslinking with CD3 mAb (1 μ g/mL UCHT1) or by the addition of 50 ng/mL phorbolmyristate acetate (PMA; Sigma) and 5 μ M ionomycin (Calbiochem, LA Jolla, CA) or of 1 μ g/mL phytohemagglutinin (Wellcome Diagnostics, Dartford, UK) for 20 hrs. Then the cells were stained with anti-CD3 and anti-CD69 mAb (clone L78; BD Biosciences), fixed, rinsed, and analyzed by flow cytometry. Cell activation was expressed as the percentage of cells that were triple-positive for the staining with B1, UCHT1, and L78 antibodies.

Intracellular Cytokine Expression

The expression of TNF- α and IL-10 by $\gamma\delta$ T cells was assessed using the Cytofix/Cytoperm Kit (BD Bioscience Pharmingen) as previously described (18). Briefly, untreated control cells and cells treated as described above for 20 hrs were permeabilized, incubated with mAbs against TNF- α or IL-10 (clones Mab11 and JES3-19F1; BD Bioscience Pharmingen) at concentrations of 0.5 μ g/ 10^6 cells, fixed, and the percentage of cytokine-positive $\gamma\delta$ T cells was determined by flow cytometry. All procedures were performed strictly according to the manufacturers' instructions.

Hsp72 Expression

PMN (10^6 cells) were incubated for 20 hrs with LPS and HS as indicated. The cells were placed on ice, and Hsp72 expression on the cell surface was assessed by incubation with 1 μ g/ 10^6 cells of anti-Hsp72 mAb (clone C92F3A-5; Stressgen, Ann Arbor, MI). Cells were fixed and analyzed by flow cytometry as described above, and the percentage of Hsp72-positive cells was determined.

PMN Killing

$\gamma\delta$ T cell-mediated cytotoxicity was determined using a Live/Dead kit from Invitrogen (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Briefly, 1 mL of PMN (1×10^6 cells) were incubated for 20 mins at 37°C with 10 μ L of a 3 mM solution of 3,3'-diiododipropylcarbocyanine [DiOC₁₈(3)]. The stained cells were washed, mixed with autologous $\gamma\delta$ T cells at a ratio of 1:5 (PMN: $\gamma\delta$ T cells), a minimal ratio resulting in significant target cell killing as optimized in preliminary experiments, and incubated for 2 hrs in a total volume of 0.2 mL in the presence or absence of 1 μ g/mL LPS or HS. Then the cells were placed on ice and stained with 10 μ g/mL propidium iodide for 10 min, rinsed, and analyzed by flow cytometry. PMN were identified as described above, and the percentage of DiOC₁₈(3)/PI double-positive cells was determined. In all experiments, samples of PMN incubated in the absence of $\gamma\delta$ T cells were included as controls to determine spontaneous cell death under the different experimental conditions.

Statistical Analysis

In the figures, each point represents a mean $\pm$ SE of individual experiments with cells from different donors evaluated only once. The error bars represent the spread of values obtained from four to seven separate experiments specified in the legends. Comparisons of differences between categories were performed with one-way analysis of variance with Neuman-Keuls *post hoc* test. Comparisons of differences within categories were performed with paired Student's *t* test with the Bonferroni correction for the number of multiple comparisons. Differences were considered significant at $p < 0.05$.

RESULTS

HS Enhances CD69 Expression of $\gamma\delta$ T Cells in the Presence of LPS

To study the effect of HS on $\gamma\delta$ T cell activation, we assessed the expression of the T-cell activation marker CD69 on the cell membrane of $\gamma\delta$ TCR-positive T cells isolated from the blood of healthy volunteers (Fig. 1). In unstimulated cells, only $8.2 \pm 0.4\%$ expressed CD69. This percentage remained unaffected by incubation of cells with LPS at concentrations of 0.01–10 $\mu\text{g/mL}$ (data not shown). HS in the absence of LPS did not induce CD69 expression (Fig. 1A). However, in the presence of 100 ng/mL LPS, clinically relevant HS levels significantly increased ($p < 0.005$) the expression of CD69 (Fig. 1A). These HS levels correspond to the hypertonicity levels reported in the blood of trauma patients who receive standard treatment with 4 mL/kg of a 7.5% NaCl solution (19), resulting in hypertonicity values ranging between 10 and 20 mM beyond isotonic values. Next, we studied how this concentration of HS affects CD69 expression of $\gamma\delta$ T cells in response to common stimuli that were added in the absence or presence of HS (Fig. 1B). CD3 crosslinking induced a significant increase in CD69 expression, which was not further changed by the addition of LPS or HS (Fig. 1B). Similarly, HS did not further increase CD69 expression in response to stimulation with phytohemagglutinin or PMA in combination with ionomycin (Fig. 1B). Since $\gamma\delta$ T cells carry out their regulatory role at sites of infection and inflammation, their ability to be readily activated by inflammatory and hypertonic stimuli may be critical for their physiologic functions in HS-treated trauma patients.

HS Inhibits the Expression of Intracellular TNF- α and IL-10 by $\gamma\delta$ T Cells

Although CD69 expression is an early event in response to T-cell activation (20), subsequent production of cytokines is an important functional outcome by which $\gamma\delta$ T cells regulate cellular immune responses. LPS induced the expression of TNF- α and IL-10 in a dose-dependent manner (Figs. 2A, C). These responses were inhibited by 20 mM HS. Next, we studied whether these inhibitory effects of HS were specific for LPS. To test this, we compared cytokine expression of $\gamma\delta$ T cells treated with LPS or with PMA plus ionomycin, anti-CD3 mAbs, or phytohemagglutinin. Clinically relevant HS concentrations significantly decreased the expression of TNF- α and IL-10 of LPS-treated $\gamma\delta$ T cells, while HS did not affect cytokine expression of cells that were stimulated with the other stimuli, suggesting that cytokine expression of $\gamma\delta$ T cells depends on signal pathways associated with toll-like receptors (TLR; Figs. 2B, D).

HS Enhances $\gamma\delta$ T Cell-Mediated Killing of PMN in the Presence of LPS

We have recently demonstrated that LPS induces the expression of Hsp72 on the cell surface of PMN, allowing $\gamma\delta$ T cells to recognize and kill inflammatory PMN (13). Here we studied if Hsp72 surface expression is affected by HS. In the absence of LPS, HS induced the expression of Hsp72 on the cell surface of PMN in a dose-dependent manner (Fig. 3). The combined stimulation of PMN with LPS (100 ng/mL) and HS further augmented the expression of Hsp72 on the cell surface of PMN (Fig. 3).

Hsp molecules are known ligands of the TCR of several $\gamma\delta$ T cell subsets (12). Since expression of Hsp on cell surfaces is critical for $\gamma\delta$ T cell-mediated killing of inflammatory phagocytes (9, 13, 21), we hypothesized that HS may enhance the killing of PMN by the upregulation of Hsp72 expression on the cell surface of PMN. To test this hypothesis, we assessed the effect of HS on the killing of purified human PMN in cocultures with autologous $\gamma\delta$ T cells.

Consistent with our previous findings (13), $\gamma\delta$ T cells purified from different donors killed 20%–25% of autologous PMN in the presence of LPS primarily within 2 hrs after initiation of the cocultures (Fig. 4A). As with CD69 expression (Fig. 1), HS by itself did not affect $\gamma\delta$ T cell-mediated killing of PMN (Fig. 4B). However, HS in the presence of LPS induced a significant increase in PMN killing of cells in cocultures with $\gamma\delta$ T cells (Fig. 5).

DISCUSSION

The present study demonstrates that clinically relevant concentrations of HS can enhance the activation of CD69 expression of $\gamma\delta$ T cells in response to LPS and their ability to kill PMN. Interestingly, HS suppressed other $\gamma\delta$ T cell responses, namely the expression of cytokines by in response to LPS stimulation. We also found that clinically relevant HS concentrations can induce the expression of Hsp72 on the cell surface of PMN and that HS augmented Hsp72 expression of LPS-stimulated PMN. Our previous work has shown that Hsp72 plays a key role in $\gamma\delta$ T cell-mediated killing of PMN, although it is possible that other agents such as microbial phospho-antigens, which induce strong $\gamma\delta$ T cell responses, may also be involved in this process. Taken together, these findings suggest that HS may act on PMN and $\gamma\delta$ T cells to augment PMN killing.

LPS did not activate CD69 expression in $\gamma\delta$ T cells and it did not affect CD69 expression of $\gamma\delta$ T cells stimulated via CD3 cross-linking or activation of protein kinase C (using PMA and ionomycin; Fig. 1). This lack of response could have several reasons. Unlike in monocytes and macrophages, the expression of TLR in $\gamma\delta$ T cells is controversial. Although TLR transcripts have been found with reverse transcriptase–polymerase chain reaction (22), attempts to demonstrate TLR molecules on the cell surface with flow cytometry have provided uncertain results (22, 23). Recent reports have shown that $\gamma\delta$ T cells respond to TLR ligands in the absence of detectable surface receptors, although it is possible that this occurred through indirect actions by cytokines or other mediators produced by contaminating monocytes (22–24). In addition, $\gamma\delta$ T cells express MD2 that is involved in LPS-CD14-TLR4 interactions (25, 26). Although the role of MD2 in the absence of TLR4 and CD14 remains enigmatic, it seems possible that this molecule may function in

association with soluble serum proteins in the culture medium. LPS responses in the absence of detectable TLR4 could also be facilitated through scavenger receptors, such as SR-B1 and CD36, which are expressed in $\gamma\delta$ T cells (reviewed in [26]). Since signals generated by these types of receptors are likely different from those produced by TLR, one could explain at least, in part, the different responses of $\gamma\delta$ T cells and monocytes to LPS.

In contrast to previous findings with conventional T cells, HS did not enhance CD69 or cytokine expression of $\gamma\delta$ T cells that were stimulated via TCR (27). This indicates that $\gamma\delta$ T cells and conventional T cells differ in the mechanisms by which HS affects signaling responses in these different cell types. Our recent work has shown that HS exerts its immunoregulatory effects through the release of cellular adenosine triphosphate that modulates cell function through autocrine feedback via purinergic adenosine triphosphate or adenosine receptors expressed on the cells surface (27, 28). Activation of some of these receptors has been shown to either suppress or enhance T-cell responses (28–30). Thus, it seems possible that differences in the distribution of purinergic receptor subtypes on $\gamma\delta$ T cells and conventional T cells could account for their response to HS (28). This suggestion is supported by data recently obtained in our laboratory demonstrating significant expression of inhibitory A2a adenosine receptors on $\gamma\delta$ T cells (unpublished results).

Combined exposure of $\gamma\delta$ T cells to LPS and HS resulted in enhanced CD69 expression (Fig. 1). It has been demonstrated that LPS can upregulate A2 adenosine receptors while down-regulating the expression of A1 and A3 receptors in macrophages, PMN, and in dendritic cells (31). Furthermore, a recent report has shown that LPS induces the overexpression of A3 receptors in human peripheral blood mononuclear cells (32). In light of these findings, it is possible that LPS may also modulate the expression of these receptors in $\gamma\delta$ T cells and that this may be a mechanism by which HS up-regulates CD69 expression. This possibility is currently under investigation in our laboratory.

Although we focused the present study mainly on clinically relevant hypertonicity, we also tested effects of higher levels of HS on $\gamma\delta$ T cell functions. Higher concentrations of HS did not affect CD69 expression (Fig. 1A), while they did markedly increase the surface expression of Hsp72 on PMN (Fig. 3), which could explain the slight and non-significant increase in PMN killing (Fig. 5). However, the exposure to 100 mM HS, a level associated with inhibition of T cell activity (27) together with LPS, inhibited $\gamma\delta$ T cell-mediated PMN killing. It seems that severe cellular distress induced by both agents overwhelms the effects of abundant Hsp expressed on the target cells.

In addition to cell-mediated killing, T cells also use cytokines to modulate immune responses. LPS treatment of $\gamma\delta$ T cells induced robust and dose-dependent increases in the expression of cytokines with pro-inflammatory (TNF- α) and anti-inflammatory (IL-10) properties. The reasons for the differences in the effects of LPS on CD69 expression and cytokine production by $\gamma\delta$ T cells are currently unclear. It seems likely that these different responses may be related to different signal pathway requirements, which are still to be defined in the case of CD69 (21). We found that HS inhibits the LPS-induced production of cytokines by $\gamma\delta$ T cells (Fig. 2). It has recently been demonstrated that manipulations of different adenosine receptors can inhibit LPS-induced cytokine production in cultured

human peripheral blood mononuclear cells and in animal experiments (33). This inhibitory potential is thought to be signaling pathway-selective, which could explain our findings that the inhibitory effects of HS on cytokine expression were seen in LPS-stimulated $\gamma\delta$ T cells, but not in cells stimulated via protein kinase C (Fig. 2B, D).

The exposure of PMN to LPS and HS resulted in a dose-dependent increase in the Hsp72 expression on the cell surface of PMN (Fig. 3). We have previously demonstrated that this LPS-induced expression on the cell surface is preceded by the *de novo* synthesis of Hsp72 and its transport to the cell membrane (13). In contrast to the well-defined roles of intracytoplasmic Hsp (reviewed in [14]), the function of externalized Hsp on the cell surface is poorly understood. It has been postulated that Hsp stabilizes plasma membranes that are damaged by toxins and in response to stress associated with infections and inflammation (34).

Hsp molecules also serve as surface markers that trigger the destruction of target cells by the immune system (reviewed in [35]). Our previous work suggests that Hsp72 plays an important role in the recognition of activated PMN, facilitating PMN clearance, and thereby limiting PMN-mediated host tissues damage. This suggests potential new strategies to protect host organs during acute inflammatory episodes. However, the feasibility of such new strategies must be tested in additional studies, as our current work is limited in its focus on LPS and Gram-negative pathogens. The questions as to whether and how products of Gram-positive microorganisms can induce Hsp expression on the cell surface of PMN and whether $\gamma\delta$ T cells are capable of recognizing activated PMN under such circumstances remain to be investigated.

The ability of HS to enhance the expression of Hsp72 on the cell surface of LPS-treated PMN suggests that the elimination of these target cells can be hastened by HS treatment. How HS augments the expression of Hsp72 on the cell surface is still unclear; to gain better insights into this important question will require additional work that is planned in our laboratory. Based on the current study, it is clear that combined signals generated by clinically relevant concentrations of HS and by LPS cause significant increases in Hsp72 expression and a corresponding increase in the ability of $\gamma\delta$ T cells to kill PMN (Figs. 4 and 5). The different cellular responses measured in our study peaked at different LPS concentrations, which had been determined through several preliminary studies. $\gamma\delta$ T cell-mediated clearance of PMN, for example, required higher concentrations of LPS, than did CD69 or cytokine expression. Most likely these differences in the responses to LPS are the result of the complex interactions of the effector and target cell-types involved. Because of their roles in host defense, PMN and $\gamma\delta$ T cells clearly differ in their sensitivities to LPS. It is surprising that treatment of $\gamma\delta$ T cell/PMN cocultures with LPS alone also increased $\gamma\delta$ T cell-mediated killing of PMN (Fig. 4B). However, unlike in our earlier observations (13), the increase in killing compared to untreated cells was not statistically significant. The reason for this difference may be related to the lower concentration of LPS used in the present study.

CONCLUSIONS

In summary, our findings demonstrate that clinically relevant concentrations of HS can significantly increase the ability of $\gamma\delta$ T cells to kill PMN when LPS is present. The predominant mechanism appears to be an increase in Hsp72 expression on the cell surface of LPS-treated PMN. These findings suggest that HS therapy may reduce PMN-induced tissue damage after major trauma and hemorrhage by facilitating the elimination of inflammatory phagocytes from host organs such as the lungs.

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Figure 1.

Hypertonic saline (HS) enhances lipopolysaccharide (LPS)-induced CD69-expression of $\gamma\delta$ T cells. *A*, Purified $\gamma\delta$ T cells were stimulated for 20 hrs with 0.1 μ g/mL LPS in the presence or absence of increasing concentrations of HS, and CD69 expression was determined by flow cytometry as described in the “Materials and Methods” section. *B*, Purified $\gamma\delta$ T cells were activated for 20 hrs by CD3 cross-linking or stimulation with 1 μ g/mL phytohemagglutinin (PHA) or 50 ng/mL phorbolmyristate acetate (PMA) + 5 μ M ionomycin (IM) in the presence or absence of 0.1 μ g/mL LPS and CD69 expression was

determined by flow cytometry. Each point represents the mean $\pm$ SE of 6 (*A*) or 4 (*B*) individual experiments with cells from different donors evaluated once; error bars represent the spread of values from separate experiments performed under identical conditions. Statistical analysis was done with one way analysis of variance followed by Neuman-Keuls *post hoc* test (between categories) or using paired Student's *t* test with Bonferroni correction for multiple comparisons (within categories).

Figure 2.

Hypertonic saline (HS) inhibits lipopolysaccharide (LPS)-induced cytokine expression of $\gamma\delta$ T cells. Expression of tumor necrosis factor (TNF)- α (A, B) and interleukin (IL)-10 (C, D) in $\gamma\delta$ T cells were measured after culture for 20 hrs in the absence (No HS) or presence of 20 mM HS (HS). Cytokine expression was determined by intracellular immune staining and flow cytometry. The cells were stimulated with increasing concentrations of LPS (A, C), or with 0.1 μ g/mL LPS, CD3 cross-linking mAbs, 1 μ g/mL phytohemagglutinin (PHA) or 50 ng/mL phorbolmyristate acetate (PMA) + 5 μ M ionomycin (IM) (B, D) in the presence or absence of 20 mM HS. Data (n = 4) and error bars were expressed as described in Figure 1. Statistical analysis was done with one-way analysis of variance followed by Neuman-Keuls *post hoc* test (between categories) or using paired Student's *t* test with Bonferroni correction for multiple comparisons (within categories).

Figure 3.

Hypertonic saline (HS) induces dose-dependent expression of heat shock protein-72 (*Hsp72*) on polymorphonuclear neutrophils (PMN). Expression of Hsp72 on the surface of PMN exposed to the indicated concentrations of HS for 20 hrs with or without 1 μ g/mL lipopolysaccharide (LPS) was determined with flow cytometry. In parallel, untreated cells, autofluorescence, and relative isotype controls were analyzed. Data ($n = 4$) and error bars were expressed as described in Figure 1. Statistical analysis was done with one way analysis of variance followed by Neuman-Keuls *post hoc* test (between categories) or using paired Student's *t* test with Bonferroni correction for multiple comparisons (within categories).

Figure 4.

$\gamma\delta$ T cells kill activated polymorphonuclear neutrophils (PMN) in coculture experiments. *A*, Killing of PMN in cocultures with or without autologous $\gamma\delta$ T cells in the presence of 1 $\mu\text{g/mL}$ lipopolysaccharide (LPS) as determined by dioctadecyloxacarbocyanine (DiOC_{18}) (3)/ propidium iodide (PI) staining and flow cytometry (details are described in the “Materials and Methods” section). *B*, The effects of LPS (1 $\mu\text{g/mL}$), hypertonic saline (HS) (20 mM) alone or in combination on the ability of $\gamma\delta$ T cells to kill autologous PMN was tested as described above. Data ($n = 7$) and error bars were expressed as described in Figure 1. Statistical analysis was done with one-way analysis of variance followed by Neuman-Keuls *post hoc* test (between categories) or paired Student’s *t* test with Bonferroni correction for multiple comparisons (within categories).

Figure 5.

At a clinically relevant level, hypertonic saline (HS) (20 mM) enhances that ability of $\gamma\delta$ T cells to kill activated polymorphonuclear neutrophils (PMN). $\gamma\delta$ T cell-mediated killing of autologous PMN in the presence or absence of 1 μ g/mL LPS and increasing concentrations of HS was assessed as described in the legend of Figure 4. Data (n = 7) and error bars were expressed as described in Figure 1. Statistical analysis was done with one-way analysis of variance followed by Neuman-Keuls *post hoc* test (between categories) or using paired Student's *t* test with Bonferroni correction for multiple comparisons (within categories).