

CHAPTER 21

HEAT SHOCK PROTEINS AND THE RESOLUTION OF INFLAMMATION BY LYMPHOCYTES

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Abstract: Depletion of phagocytes that infiltrate host organs like the lungs reduces inflammatory damage to tissues. Understanding the mechanisms by which this process occurs could lead to new therapeutic approaches to limit the detrimental effects of inflammation. The lungs, gastrointestinal tract, and skin are particularly prone to infection. Specialized immune cells protect these organs from tissue damage by eliminating phagocytes from inflamed tissues by recognizing signals produced by the phagocytes. One such signal is heat shock proteins (HSP) expressed on the cell surface of phagocytes. These HSP closely resemble their microbial equivalents, and therefore phagocytes that are labeled by HSP are recognized as target cells. T lymphocytes bearing $\gamma\delta$ T cell receptor (TCR) elicit fast responses to invading pathogens. Since the $\gamma\delta$ TCR has limited germline-encoded diversity, HSP are an ideal target for recognition by these cells. $\gamma\delta$ T cells exert cytotoxic actions towards macrophages and neutrophils that express Hsp60 or Hsp70, respectively, on their cell surface. Through the recognition of HSP on the cell surface of inflamed cells, $\gamma\delta$ T cells eliminate phagocytes from inflammatory sites, thereby preventing host tissue damage

Keywords: $\gamma\delta$ TCR, T lymphocytes, macrophages, neutrophils, inflammatory tissue damage, immunoregulation

INTRODUCTION

Trauma, hemorrhagic shock, and sepsis result in a systemic inflammatory response syndrome (SIRS) that impairs the function of host organs by an uncontrolled and destructive immune response that results in severe tissue damage (Rangel-Frausto et al., 1995). Inflammatory damage to critical organ systems (e.g., vasculature, lungs, and liver) is a major cause of post-traumatic morbidity and mortality of intensive care patients. Despite extensive scientific efforts, the mechanisms involved in the initiation, development, and resolution of inflammatory host organ damage are not completely understood. This is one of the reasons that limit the successful

treatment of trauma casualties and patients with sepsis, despite of the broad range of therapeutic modalities available today (Abraham, 1997). Although the specific events leading to the loss of regulation of inflammatory reactions are not known, it is clear that acute lung injury (ALI) is associated with an accumulation and activation of various inflammatory cells within the lungs (Downey et al., 1999). Neutrophils (PMN) play a prominent role in lung injury. The uncontrolled and self-perpetuating pulmonary inflammation seen in ALI can result in full-blown acute respiratory distress syndrome (ARDS). Most patients with ARDS require mechanical ventilation and at least 50% of these patients die in the intensive care unit, usually due to multiple organ system failure (MOSF), nosocomial infections of the injured lungs, and severe sepsis (Haslett et al., 2000). The mechanisms by which the host down-regulates inflammatory reactions to limit the risk of ALI and ARDS are not fully understood. Moreover, the control systems that coordinate the activities of the various immune cell types within inflamed tissue are incompletely characterized. Recent advances in our understanding of these mechanisms may lead to the development of novel therapeutic approaches to prevent acute inflammatory organ injury in trauma casualties and in sepsis patients.

RESOLUTION OF THE ACUTE INFLAMMATORY PROCESS

Although the rapid initiation of the protective immune response to invading pathogens is critically important to protect the host from infectious agents, it is equally important to terminate this immune response in order to protect host tissues from the harmful effects of prolonged exposure to the toxic mediators released from inflammatory cells. Clear evidence for the importance of this balanced immune response can be observed during the course of pulmonary inflammation secondary to trauma or sepsis. The initial moderate inflammatory reactions seen in the lungs can derail, become self-destructive, and can ultimately develop into lethal ARDS. Similarly, other clinical conditions such as tuberculosis, asthma, and glomerulonephritis are associated with a failure of the cellular immune response to terminate its inflammatory cascades, ultimately leading to chronic disease characterized by extensive tissue damage and scarring that can seriously impair organ system functions (Carding and Egan, 2000; Haslett, 1995, 1999). The resolution of the inflammatory immune response coincides with the normalization of vascular tone and permeability, drainage of edema fluid, and the clearance of activated immune cells recruited to affected tissues (Haslett, 1995, 1999). Since vascular components of inflammation, such as increased vascular permeability and edema are primarily modulated by inflammatory mediators (i.e., amines, eicosanoides, nitric oxide, cytokines, etc.), which are of cellular origin, it is clear that the pivotal mechanism leading to the resolution of acute inflammation is based on the inactivation and elimination of inflammatory cells sequestered to inflamed tissues.

Macrophages ($M\Phi$) are able to effectively eliminate inflammatory cells by phagocytic ingestion, once they recognize target cells through appropriate signals (Melley et al., 2005; Savill, 1997). Target cells may provide such signals through functional

or structural mechanisms such as the release of chemical mediators or the expression of cell surface markers. One such mechanism is based on changes of the cellular phenotype, for example due to the termination of specific cellular functions or due to programmed cell death, which facilitate phagocytosis of target cells by M Φ . Such mechanisms have been demonstrated in several systems that drive the elimination of PMN or lymphocytes from inflamed tissues (Cox et al., 1994; Haslett, 1999; Savill, 1997). The involvement of activation-induced apoptosis in the clearance of immune cells was primarily described for the inactivation of T lymphocytes as a result of Fas/Fas ligand interplay (Nagata, 1997). In a similar manner, ligation of Fas or TNF- α receptors on the cell surface was shown to induce apoptosis of PMN (Matsumoto et al., 1995; Murray et al., 1997).

Phagocytosis of apoptotic immune cells by M Φ is not the universal mechanism by which inflammatory cells are cleared from inflamed tissue. Many inflammatory mediators (e.g., LPS, chemotactic peptides, prostaglandins) are known to inhibit PMN apoptosis, which would prevent subsequent clearance of PMN through phagocytosis by M Φ (Lee et al., 1993; Rossi et al., 1995; Whyte et al., 1993). Moreover, it is not clear how M Φ themselves would be eliminated upon completion of their task. The mechanisms responsible for the down-regulation and final elimination of M Φ are poorly understood. Although, similar to PMN, M Φ have been shown to undergo apoptosis in response to various stimuli *in vitro* (Khelef et al., 1993; Mangan and Wahl, 1991), it is not clear if this process also occurs *in vivo* (Bellingan et al., 1996), and if so, how it is regulated. However, such mechanisms must exist, since the enormous influx of activated PMN, lymphocytes, and M Φ in response to microbial or other inflammatory challenges is usually cleared from affected tissue without residual tissue damage. Conversely continued accumulation of large amounts of M Φ is a hallmark feature of chronic microbial and autoimmune inflammatory diseases (Egan and Carding, 2000). Given the complexity of the issue, there is an assumption that inflammatory cells express signals alternative to apoptosis inducing non-phagocytic mechanisms that lead to their inactivation and elimination. One possible signaling pattern is the externalization of stress proteins, which are exhibited upon abnormal cellular homeostasis, and thereby appeal to the host's immune surveillance. An example of this mechanism is the recent observation that surface-expressed heat shock proteins (HSP) are recognized by natural killer (NK) cells (Multhoff et al., 1997).

HEAT SHOCK PROTEINS AS STRESS RESPONSE MARKERS

Heat shock protein (HSP) molecules comprise a family of cellular proteins that perform important housekeeping functions (Zugel and Kaufmann, 1999; Zwierska et al., 2006). Under normal conditions, cells constitutively produce several different HSP family members that contribute to the regulation of cell homeostasis. The major chaperone functions of intracellular HSP are related to protein folding, transport, and repair (Hartl, 1996; Schlesinger, 1990). HSP are also involved in intracellular signal transduction (Pratt and Toft, 1997). Under various pathologic conditions both

prokaryotic and eukaryotic cells increase intracellular expression of HSP in order to protect the cells from stress, primarily by preventing uncontrolled protein unfolding induced by stressful agents (Parsell and Lindquist, 1993; Zigel and Kaufmann, 1999). Members of the Hsp70 family, for example, have been shown to protect cells from oxidative injury and metabolic stress like ethanol intoxication (Dastoor and Dreyer, 2000; Su et al., 1998). Transgenic mice expressing high levels of Hsp70 revealed increased resistance to ischemic myocardial injury (Radford et al., 1996), while overexpression of Hsp70 in transfected cells conferred resistance to TNF- α (Jaattela et al., 1992). HSP are typical cytosolic proteins that lack the specific leader sequence usually required for expression on the cell surface. Nevertheless, several studies have demonstrated cell surface expression of HSP. Hsp60 was detected on the surface of Daudi cells, a human B cell lymphoma cell line (Fisch et al., 1990; Kaur et al., 1993). Hsp70 was found on Daudi cells and H9 cells, a T cell lymphoma cell line (Di Cesare et al., 1992), while Hsp90 was identified on the cell surface of cells in human solid tumors (Ferrarini et al., 1992). Other studies identified HSP on non-transformed cells: surface expressed Hsp60 was identified on M Φ (Wand-Wurttenberger et al., 1991) and endothelial cells (Xu and Wick, 1993), while Hsp70 and Hsp90 products were found on B cells (Vanbuskirk et al., 1989), monocytes (Erkeller-Yuksel et al., 1992), and PMN (Hashiguchi et al., 2001). The mechanisms involved in the translocation of HSP to the cell surface are poorly understood. In several reports, this phenomenon was attributed to apoptotic transformation of the cells (Di Cesare et al., 1992; Poccia et al., 1993). Immuno-precipitation experiments revealed that HSP molecules expressed on the cell surface contain their entire amino acid structures (Ferrarini et al., 1992; Kaur et al., 1993), however crucial questions as to whether these HSP expression requires *de novo* synthesis or not remain to be addressed. Since HSP are known to have strong protein-binding capacity, it is reasonable to suggest that some HSP members may be transported to the cell surface and bound to membrane proteins such MHC products (DeNagel and Pierce, 1992). Alternatively, lipid anchorage of HSP molecules to the cell surface may be an additional mechanism that cannot be ruled out.

In contrast to the well defined roles of intracellular HSP, the functions of HSP proteins expressed on the surface of normal (Erkeller-Yuksel et al., 1992; Ishiyama et al., 1996), infected (Di Cesare et al., 1992), transformed (Ferrarini et al., 1992; Hantschel et al., 2000; Multhoff et al., 1997; Multhoff and Hightower, 1996), and apoptotic cells (Poccia et al., 1996) are poorly understood. Several investigators have indicated that surface HSP can serve as markers of a cell's commitment to undergo elimination by the innate immune surveillance system (Carding and Egan, 2000; Egan and Carding, 2000; Hirsh et al., 2006; Multhoff et al., 1997; Multhoff and Hightower, 1996; Roigas et al., 1998). The mechanisms that lead to the translocation of HSP to the cell surface may be related to the ability of HSP to stabilize plasma membranes (Torok et al., 1997). Cells may use HSP to reinforce membranes destabilized by toxins and stress-related metabolites the cells encounter during episodes of infection or inflammation. The functions of membrane-associated HSP may not be restricted to membrane and protein stabilization. Rather, cell surface

localization of HSP may represent an intermediate step in the secretion of HSP into the extracellular space (Hightower and Guidon, 1989; Multhoff and Hightower, 1996). However, the release of HSP in the extracellular environment can also be the result of cell destruction and the release intracellular content from damaged cells (Basu et al., 2000). Although several HSP members have been detected in the extracellular milieu, the detailed mechanisms responsible for the secretion of HSP and the exact functions of extracellular HSP have not been defined.

HEAT SHOCK PROTEINS AS TARGETS FOR IMMUNE CELLS

Once HSP appear on the cell surface, they are accessible to the immune surveillance system. HSP expressed on cell surfaces can elicit strong immune responses, as these molecules contain several highly conserved epitope sequences with strong immunogenic properties (Kaufmann and Schoel, 1994; Shinnick, 1991; Zugel and Kaufmann, 1999). On the other hand, an overzealous immune response to HSP can have several undesirable effects. Due to high degree of phylogenetic conservation, HSP species of microbial origin and HSP molecules produced by stressed host cells have similar immunogenic properties (Zugel and Kaufmann, 1999). Moreover, abundantly expressed HSP undergo processing by antigen presenting cells, and cells expressing HSP alone or presenting them in the context of MHC molecules are recognized by immune cells as potential targets of self-reactive antibodies or lymphocytes with specificity for HSP (Kaufmann and Schoel, 1994; Zugel and Kaufmann, 1999). In a number of autoimmune disorders in humans and animal models, HSP expression by affected cells has been observed, supporting the concept that HSP expression contributes to immunopathologic changes associated with autoimmune diseases. For example, a significant proportion of patients with systemic lupus erythematosus (SLE) expressed Hsp90 on lymphocytes and monocytes (Erkeller-Yuksel et al., 1992). Similarly, in MRL/lpr mice, a strain used in animal models of SLE, increased surface Hsp90 localization and antibodies against Hsp90 have been found (Faulds et al., 1994; Latchman and Isenberg, 1994). In these two systems, the overexpression of Hsp90 and the presence of antibodies provide tentative evidence that this molecule serves as an autoantigen, possibly causing the autoimmune alterations in SLE. Another example of HSP cell surface localization has been described for lesions in chronic experimental autoimmune encephalomyelitis (Gao et al., 1995).

$\gamma\delta$ T LYMPHOCYTES – MAJOR RESPONDERS TO HEAT SHOCK PROTEINS

Upon recognition of HSP expressed on the cell surface, the immune system proceeds to eliminate the pathogen-associated antigen by the destruction of the HSP bearing cell. This mechanism may be relevant for the clearance of infected or transformed cells as well as for the elimination of inflammatory cells, which may be a key event in the termination of acute inflammation.

T lymphocytes bearing the $\gamma\delta$ heterodimer of the T cell receptor ($\gamma\delta$ T cells) have been implicated in the regulation of the host defense against microbial invaders and in the response to inflammatory challenges (Born et al., 1999; Cai and Tucker, 2001). The regulatory function of $\gamma\delta$ T cells is mediated through the production of cytokines such as IFN- γ , IL-10, and TNF- α as well as through direct cytotoxicity towards target cells (Cai and Tucker, 2001; Calandra and Heumann, 2000). Accumulation of $\gamma\delta$ T cells is seen at sites of inflammation associated with intracellular bacterial, viral, and parasitic infections. Moreover, in experiments with mice that lack $\gamma\delta$ T cells, bacterial infections culminate in an abnormally exaggerated and prolonged pro-inflammatory response and in increased mortality (D'Souza et al., 1997; Saunders et al., 1998; Tam et al., 2001). These findings suggest that $\gamma\delta$ T cells protect host tissues from inflammatory injury (Hirsh et al., 2004b).

Like conventional $\alpha\beta$ T lymphocytes, $\gamma\delta$ T cells can recognize antigen in the context of MHC molecules, but the majority of $\gamma\delta$ T cells employ a strategy of recognition of multiple surface molecules that present antigens such as classical MHC gene products (Schild et al., 1994; Weintraub et al., 1994) or alternative constitutive and stress-induced MHC class I-related molecules (Groh et al., 1998; Kim et al., 1995; Porcelli et al., 1989; Soloski et al., 1995). In addition, $\gamma\delta$ T cells appear to directly recognize antigens expressed on the surface, as this recognition is independent of antigen processing and presentation by MHC molecules (Li et al., 1998). These specialized mechanisms allow $\gamma\delta$ T cells to rapidly respond to invading pathogens. However, due to a limited germline-encoded diversity of the $\gamma\delta$ TCR (Hayday, 2000), this response is not very specific, and thus, conserved ligands such as HSP seem to be ideal targets for recognition by $\gamma\delta$ T cells. In fact, peptides derived from bacterial HSP are ligands well-characterized for their ability to activate $\gamma\delta$ T cells (O'Brien et al., 1992). Experimental listeriosis, for example, specifically activates $\gamma\delta$ T cells that are reactive for Hsp60 and Hsp70, and these cells may have a protective role in the immunity against infection (Hiromatsu et al., 1992; Kimura et al., 1996; Kimura et al., 1998). Similarly, in the mouse model of malaria, Hsp60 reactive $\gamma\delta$ T cells adoptively transferred into recipients conferred partial protection against the parasites (Tsuji et al., 1994). Hsp70 reactivity among $\gamma\delta$ T cells has been described as well (Beagley et al., 1993). In summary, HSP expressed on the cell surface is recognized by the immune system as a pathogen-associated molecular pattern that requires the induction of a cytotoxic immune response (Janeway, 1992; Zügel and Kaufmann, 1999).

This cytotoxic immune response is more widely utilized than to just eliminate cells infected with intracellular pathogens. It may also be directed at cells that exhibit HSP following transformation into cancer cells. Carcinogenesis usually includes several genetic and phenotypic changes, which render cancer cells sensitive to an immune attack. A well known mechanism of tumor surveillance involves NK cells that kill tumor cells without the need for antigenic specificity (Lanier, 1997). The cytotoxic activity of NK cells depends on antibodies bound to surface structures of the target cell or on the recognition of abnormal MHC class I molecules (Kos and Engleman, 1996; Lanier, 1997). HSP molecules have also been shown to be

involved in NK cell cytolytic activity. Specifically Hsp70 has been revealed as a targeting molecule of NK cells (Gastpar et al., 2004; Multhoff et al., 1997) that stimulates both cytotoxicity and migration in a concentration-dependent, highly selective, and chemokine-independent manner (Gastpar et al., 2004). Hsp60 is also involved in the regulation of NK cell cytotoxicity by interfering with the inhibitory CD94/NKG2A complex (Michaelsson et al., 2002). It has been demonstrated that human, murine, and rat $\gamma\delta$ T cells transcribe NK-associated genes and possess NK-like cytolytic activities (Dyugovskaya et al., 2003; Fahrner et al., 2001; Hirsh et al., 2004b; Laad et al., 1999). Hence, although freshly isolated $\gamma\delta$ T cells usually do not express NK cell surface markers (Born et al., 1999), these cells possess NK-like cytotoxic activity.

It was recently found that $\gamma\delta$ T cells exert cytotoxicity against activated macrophages, suggesting the involvement of $\gamma\delta$ T cells in terminating inflammation and in facilitating the recovery from infections (Carding and Egan, 2000; Egan and Carding, 2000). Infectious challenges strongly induce the expression of Hsp60 on the external cell membrane of M Φ (Belles et al., 1999), which significantly increases the susceptibility of M Φ to an attack by $\gamma\delta$ T cells (Carding and Egan, 2000; Egan and Carding, 2000). It has also been suggested that HSP-specific $\gamma\delta$ T cells interact directly with Hsp60 expressed on the cell surface, which is similar to the interaction of $\gamma\delta$ T cells with cancer cells (Fisch et al., 1990; Kaur et al., 1993; Laad et al., 1999). The recognition of cells expressing Hsp60 could be significantly inhibited in these reports by pretreatment of the target cells with Hsp60-specific antibodies (Fisch et al., 1990; Kaur et al., 1993). Thus, Hsp60 plays a significant role in the recognition and destruction of M Φ after their exposure to pathogens. However, to date no data are available on the role of other HSP family members in this process that results in the clearance of activated M Φ and perhaps other inflammatory cells.

SURFACE EXPRESSION OF HSP FACILITATES KILLING OF INFLAMMATORY PMN BY $\gamma\delta$ T CELLS

Activated PMN play a central role in the damage of lung tissue in sepsis (Downey et al., 1999; Hirsh et al., 2004a; Windsor et al., 1993). However, the mechanisms that control the lethal actions of PMN and diminish their damaging effects on lung tissues in individuals who recover from sepsis are unknown. We recently demonstrated that $\gamma\delta$ T cells can recognize and eliminate PMN and that the recognition of inflammatory PMN activated by lipopolysaccharides depends on the expression of Hsp70 on the cell surface of the PMN (Hirsh et al., 2006).

LPS Induces Hsp60 and Hsp70 Expression in PMN

Gram-negative bacteria are the main cause of sepsis in the animal model used in our study. LPS (lipopolysaccharides) are products of gram-negative bacteria that are involved in the initiation of the inflammatory response to infection (Bone, 1991).

The mammalian immune system recognizes LPS as a danger signal that activates M Φ and PMN (Janeway, 1992). LPS has been shown to induce the expression of HSP by M Φ (Kim et al., 1999). Because of the central role of PMN in the development of lung tissue damage in sepsis, we studied whether LPS can also induce HSP expression by PMN. We isolated human PMN from healthy volunteers, exposed them to LPS, and measured the expression of Hsp60 and Hsp70 on the cell surface of the PMN (Figure 1A, B).

LPS exposure resulted in the rapid and dose-dependent expression of Hsp70 that reached a maximum within 30 min after the stimulation. By contrast, the expression

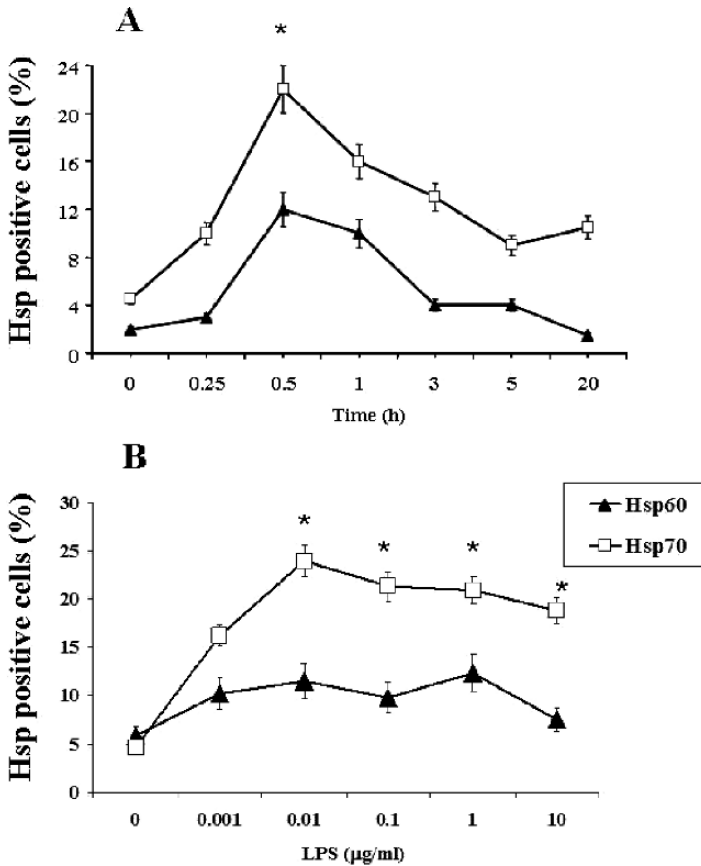


Figure 1. Heat shock protein expression by PMN. A. The time course of cell surface expression of Hsp60 and Hsp70 was determined at different time points after the stimulation of PMN with 1 $\mu\text{g/ml}$ LPS. The data shown are aggregate average of 3 experiments with cells from different donors (mean \pm SD). B. Isolated PMN were exposed to the indicated LPS concentrations and surface Hsp expression was determined after 60 min (data are aggregate averages of cells from 3 different donors; mean \pm SD). * $p < 0.02$ compared to untreated cell (0)

of Hsp60 on the cell surface seemed delayed and less pronounced than that of Hsp70. Maximum surface expression of Hsp70 was more than twice as high as that of Hsp60. After overnight incubation with LPS, surface expression of Hsp70 but not of Hsp60 was still significantly elevated compared to untreated controls (Figure 1A). These findings show that the inflammatory stress induced by LPS causes rapid and sustained Hsp70 expression on the cell surface of PMN.

Sepsis Induces Hsp70 Expression by PMN Sequestered into the Lungs

Using a mouse model of cecal ligation and puncture (CLP) to induced sepsis (Hirsh et al., 2004a; Hirsh et al., 2004b; Hirsh et al., 2006), we tested if sepsis induces Hsp70 surface expression *in vivo*. Mice were subjected to CLP and surface Hsp70 on the PMN sequestered to the lungs and on PMN in the peripheral blood was determined after 24 h with flow cytometry. We found that $17.2 \pm 2.9\%$ of the PMN in lung tissues of septic mice were Hsp70-positive, while only $5.6 \pm 1.5\%$ of the PMN of sham-operated control animals without sepsis expressed Hsp70 ($p < 0.002$). Interestingly, Hsp70 expression of PMN in the peripheral blood of septic animals was negligible and did not differ from that of sham controls (0.4 ± 0.1 vs. $0.5 \pm 0.2\%$). These findings were consistent with the assumption that $\gamma\delta$ T cells in the lungs of septic animals may be able to eliminate inflammatory PMN through interactions through Hsp70 expressed on the cell surface of the PMN.

Hsp70 Surface Expression Requires *de novo* Protein Synthesis

Comparison of the time courses of intracellular vs. extracellular HSP expression of human PMN stimulated with LPS suggested that *de novo* protein expression and protein translocation to the cell membrane is required for the expression of Hsp60 and Hsp70 on the cell surface. In order to determine if HSP expression on the cell surface of PMN involves protein transcription, translation, and protein transport, we treated isolated human PMN with inhibitors that interfere with these different processes. The inhibitors of transcription (actinomycin D), translation (cycloheximide), and protein transport (brefeldin A) caused a significant suppression of Hsp70 expression on the cell surface of LPS-stimulated PMN (Figure 2). The same inhibitors were less effective in preventing the expression of Hsp60 and only brefeldin A was able to significantly reduce Hsp60 expression by LPS-stimulated PMN. These data indicate that LPS-stimulation of Hsp70 expression on the cell surface of PMN requires *de novo* protein synthesis.

$\gamma\delta$ T Cells Kill LPS-treated PMN by Direct Cell-to-Cell Contact that Involves Hsp70

The data shown above indicated that LPS induces rapid expression predominantly of Hsp70 on the cell surface of PMN. Together with our recent report (Hirsh et al., 2006), our data suggest that Hsp70 is involved in the interaction of $\gamma\delta$ T cells

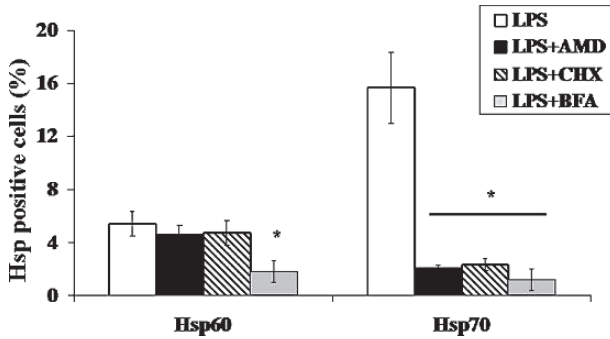


Figure 2. Up-regulation of Hsp70 cell surface expression by LPS depends on de novo protein synthesis. PMN were incubated for 1 h with LPS (1 $\mu\text{g}/\text{ml}$) in the presence or absence of actinomycin D (AMD; 2 $\mu\text{g}/\text{ml}$), cycloheximide (CHX; 5 $\mu\text{g}/\text{ml}$), or brefeldin A (BFA; 2 $\mu\text{g}/\text{ml}$) and cell surface expression of Hsp60 were determined by flow cytometry and expression of Hsp60 and Hsp70 were determined by flow cytometry and expressed as the percentage of Hsp-positive PMN. * $p < 0.05$ vs. LPS alone

with LPS-stimulated PMN. To test if $\gamma\delta\text{T}$ cells can kill LPS-stimulated PMN, we co-cultured isolated human PMN with autologous $\gamma\delta\text{T}$ cells and measured PMN killing using propidium iodide uptake. Co-cultures with $\alpha\beta\text{T}$ cells or PMN alone served as controls.

As shown in Figure 3A, co-culture with $\alpha\beta\text{T}$ cells over a period of 20 h did not increase the death of PMN. After incubation overnight, the killing of PMN in the presence of $\gamma\delta\text{T}$ cells was significantly enhanced when compared to the $\alpha\beta\text{T}$ controls ($p < 0.021$) or to controls in the absence of T cells, representing spontaneous cell death of PMN ($p < 0.009$). The exposure of cultured cells to LPS significantly ($p = 0.038$) increased $\gamma\delta\text{T}$ cell-mediated PMN killing (Figure 3A). The majority of target cells were killed by $\gamma\delta\text{T}$ cells within the first 2 h after co-culture (Figure 3B).

These findings show that $\gamma\delta\text{T}$ cells but not $\alpha\beta\text{T}$ cells efficiently kill LPS stimulated PMN, suggesting that $\gamma\delta\text{T}$ cells may serve to eliminate inflamed PMN from inflammatory sites such as the lungs during sepsis. In order to gain more insight into the cellular mechanisms by which $\gamma\delta\text{T}$ cells target LPS-stimulated PMN, we performed real-time imaging experiments of human PMN in co-culture with autologous $\gamma\delta\text{T}$ cells. These cells were placed in the stage micro-incubator of an inverted microscope and time-lapse images were taken after the addition of 1 $\mu\text{g}/\text{ml}$ LPS. We observed a number of highly mobile $\gamma\delta\text{T}$ cells that interacted with multiple groups of sedentary PMN through filopodia- and pseudopodia-like protrusion produced by the $\gamma\delta\text{T}$ cells. Shortly after the direct cell-to-cell contact between the PMN and these protrusions of the $\gamma\delta\text{T}$ cells, the PMN swelled and the targeted PMN disintegrated within 10 min. These observations demonstrate that $\gamma\delta\text{T}$ cells are highly effective in locating and killing LPS-stimulated PMN through direct cell contact between the effector and target cells. This process is rapid and the number of PMN killed closely corresponded to the percentage of PMN that express Hsp70 on the cell surface after LPS-stimulation. As discussed above, HSP

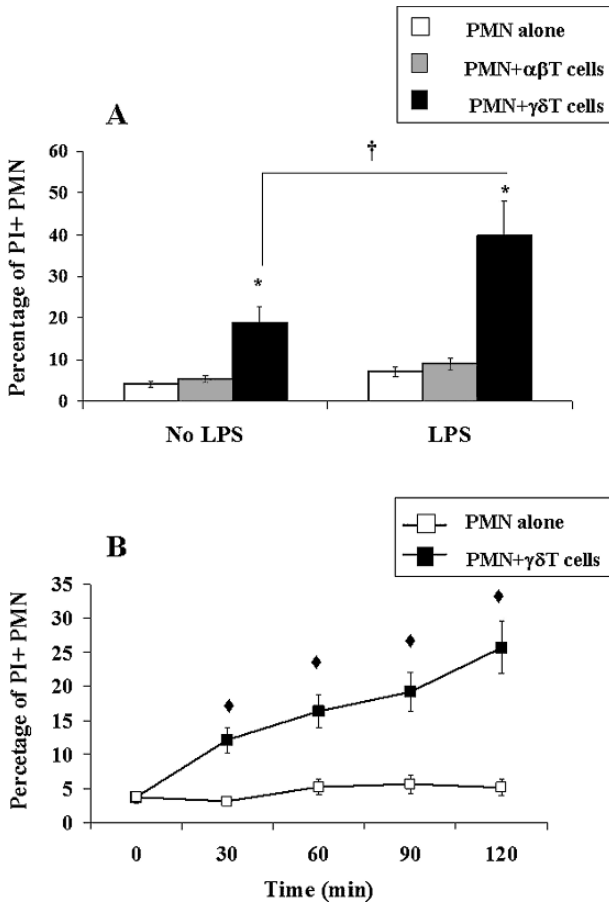


Figure 3. Killing of PMN by $\alpha\beta$ T and $\gamma\delta$ T lymphocytes. Autologous PMN co-cultured in the absence or presence of 1 $\mu\text{g/ml}$ LPS with $\alpha\beta$ T or $\gamma\delta$ T lymphocytes for 20 h (A) or for the indicated time periods (B). PMN killing was determined as percentage of propidium iodide (PI)-positive PMN using flow cytometry (n=5), and compared with the rate of spontaneous cell death. * $p < 0.025$ compared to $\alpha\beta$ T cells; † $p = 0.038$ compared to untreated cells; ♦ $p < 0.05$ compared PMN alone

are known ligands of $\gamma\delta$ T cell receptors, suggesting that Hsp70 on the cell surface of PMN could serve to facilitate the targeting and killing of LPS-stimulated PMN by $\gamma\delta$ T cells. To test this possibility, we treated PMN with neutralizing antibodies to Hsp70 or with an unrelated isotypic control immunoglobulin preparation. Then the cells were co-cultured overnight with $\gamma\delta$ T cells in the presence of 1 $\mu\text{g/ml}$ LPS and PMN killing was assessed using the PI uptake method described above. We found that treatment with Hsp70 antibodies but not with isotypic control IgG caused a significant reduction of PMN killing from $16 \pm 3\%$ to $5 \pm 2\%$ ($p = 0.012$).

These data show that Hsp70 displayed on the cell surface of LPS-stimulated PMN is required for $\gamma\delta$ T cell-mediated killing of inflammatory PMN.

CONCLUDING REMARKS

Due to specialized patterns of antigen recognition and activation (Hayday, 2000), $\gamma\delta$ T cells are capable of fast and vigorous responses to various pathogens. Conventional $\alpha\beta$ T lymphocytes need 5–7 days to manifest their cytotoxic activity (Born et al., 1999; Cai and Tucker, 2001) and thus it is not surprising that self-tolerant clones of circulating $\alpha\beta$ T lymphocytes do not recognize autologous PMN as targets. The cytotoxic activity of $\gamma\delta$ T cells towards activated macrophages has been recognized as an important feature leading to the down-regulation of inflammatory reactions (Carding and Egan, 2000; Egan and Carding, 2000). Several clones of $\gamma\delta$ T cells have been shown to be able to kill activated macrophages that express HSP on their cell surface (Egan and Carding, 2000). Hence, the LPS-induced expression of Hsp70 on the cell surface of PMN suggests that $\gamma\delta$ T cells utilize a similar mechanism to recognize and eliminate inflammatory PMN for example from lungs of patients during recovery from a septic episode. Increased PMN cell surface expression of Hsp70 has been reported in critically ill patients who suffer from trauma, endotoxemia, and sepsis (Hashiguchi et al., 2001; Kindas-Mugge et al., 1993). Moreover, we found that surface Hsp70 expression on PMN sequestered into the lungs of mice after experimental sepsis was increased. Although there are clear differences in the phenotypes of the $\gamma\delta$ T cell subpopulations in human blood and the lungs of mice, our previous works (Hirsh et al., 2004b; Hirsh et al., 2006) and the data shown by other investigators (Wang et al., 2001) suggest that $\gamma\delta$ T cell populations in both species may have functional similarities in their abilities to recognize and kill Hsp70-bearing PMN. However, future studies will be needed to determine if the killing of PMN by $\gamma\delta$ T cells *in vivo* indeed requires target cell recognition through surface Hsp70 expression.

LPS-induced expression of Hsp60 and Hsp70 in PMN differed with regard to the timing of cell surface expression (Figure 1). The relatively slow externalization of Hsp60 and its sensitivity to brefeldin A, an inhibitor of protein processing and transport in the Golgi complex, suggest posttranslational modifications of the existing protein before the expression on the cell surface (Ripley et al., 1993). Under identical conditions, LPS induced rapid Hsp70 expression in the cytoplasm and on the cell membrane of PMN. This process was blocked by a broad range of inhibitors suggesting *de novo* synthesis of Hsp70. Similarly rapid appearance of newly synthesized Hsp70 also has been demonstrated in LPS-stimulated macrophages (Kim et al., 1999).

The data suggest that Hsp70 could serve as an important regulatory signal that prompts immunological attack by $\gamma\delta$ T cells of Hsp70-bearing PMN (Figure 4). We could confirm the direct involvement of cell surface expression of Hsp70 in $\gamma\delta$ T cell-mediated killing of LPS-treated PMN with the use of neutralizing anti-Hsp70 antibodies. While antibodies to Hsp70 blocked $\gamma\delta$ T cell-mediated PMN killing, no

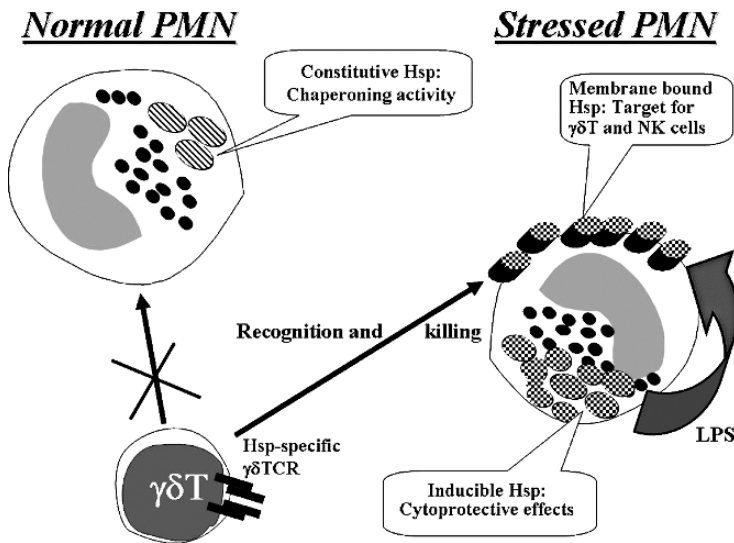


Figure 4. Under normal conditions Hsp70 synthesized by PMN are located in the cytoplasm and not recognized by $\gamma\delta T$ cells with Hsp70-specific TCR. Under stress conditions, e.g. during inflammation, PMN express increased levels of Hsp70 that rapidly translocates to the cell

such blocking effect was seen with antibodies to Hsp60. These findings suggest that Hsp60 plays only a minor, if any role in mediating the killing of LPS-stimulated PMN, although Hsp60 appears to have a more significant role in the clearance of other target cells, i.e. M Φ by $\gamma\delta T$ cells (Egan and Carding, 2000; Laad et al., 1999).

Inhibition of the recruitment of inflammatory cells to host organs such as the lungs, gut, and liver can successfully control the detrimental consequences of inflammatory responses due to host tissue damage. Another effective protective mechanism is the inactivation and depletion of inflammatory cells that are sequestered to these host tissues. The results of our study complement previous reports on the important protective functions of $\gamma\delta T$ cells in host protection. Increased $\gamma\delta T$ cell levels in the lungs of animals with sepsis were paralleled by decreased PMN counts, diminished tissue damage, and improved survival (Hirsh et al., 2004b; Hirsh et al., 2006). The majority of the $\gamma\delta T$ cells in the lungs were of the V δ 4-subtype, suggesting intestinal origin (Cai and Tucker, 2001) while the portion of other $\gamma\delta T$ cell subpopulations usually found in healthy lungs or lymphoid organs (V γ 2V δ 5/6 and V γ 1.2V δ 5/6) was comparatively minor. These findings are consistent with reports suggesting that mice deficient of $\gamma\delta T$ cells exhibit abnormally strong inflammatory responses to various infectious insults. These animals show extensive tissue necrosis, delayed resolution of inflammatory infiltrates, and increased overall mortality (Carding and Egan, 2000; D'Souza et al., 1997; Griffin et al., 1991; Tam et al., 2001). Taken together, these findings suggest that the presence of $\gamma\delta T$ cells at inflammatory sites is essential to control the inflammatory response and to protect host tissues from collateral damage caused during episodes of fierce immune defense.

Besides their ability to eliminate inflammatory cells, $\gamma\delta$ T cells possess additional mechanisms that allow them to protect host tissues and organ integrity. For example, $\gamma\delta$ T cells produce anti-inflammatory cytokines such as IL-10 (Hirsh et al., 2004b; Schaible et al., 1999) that inhibit the production of pro-inflammatory mediators including IL-1, TNF- α , and IL-8 generated by inflammatory cells within affected tissues. Through such indirect mechanisms, $\gamma\delta$ T cells may reduce the recruitment of additional PMN to sites of inflammation, suppress oxidative burst and degranulation, and thereby further diminish the risk of PMN-mediated tissue injury (Calandra and Heumann, 2000; Capsoni et al., 1997). These additional mechanisms of action in combination with the ability of $\gamma\delta$ T cells to kill inflammatory Hsp70-positive PMN (Figure 4) may explain the powerful role of $\gamma\delta$ T cells in limiting inflammatory processes and preventing host tissue damage.

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