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CELLULAR ACTIVATION MECHANISMS IN SEPTIC SHOCK

Jocelyn S. Downey and Jiahuai Han

Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037

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1. ABSTRACT

Septic shock is an increasingly important clinical condition, characterized by systemic hypotension, ischemia, and ultimately organ failure. In Gram negative infection, the bacterial cell wall component, lipopolysaccharide (endotoxin, LPS), has been strongly linked to the pathophysiological responses that result in septic shock. LPS is bound in plasma to a protein called LPS-binding protein (LBP), which facilitates the binding of LPS to a cell surface receptor, CD14. Binding to CD14 stimulates cell signaling mechanisms that result in the production of inflammatory cytokines. However, the events which follow LPS binding to CD14 and which lead to the production of cytokines remain unclear. It has recently become evident that a number of phosphorylation cascades including MAP kinase pathways and NF-κB activation pathway are initiated by exposure of cells to LPS. These cascades act at both the transcriptional and translational levels to regulate cytokine production. This review will focus on the signaling pathways that are initiated by LPS and the cellular effects of the signaling pathways.

2. INTRODUCTION

Septic shock is a systemic inflammatory response associated with around 100,000 deaths annually in the United States, characterized by fever, hypotension, disseminated intravascular coagulation (DIC), and multi-organ failure (1). Infections by Gram positive bacteria and fungi as well as Gram negative organisms, can result in septic shock. In Gram negative disease, lipopolysaccharide (LPS) is strongly implicated in the physiological responses resulting from sepsis (2). Cells of the immune system respond to LPS through a pathway involving a plasma-protein, named LPS-binding protein (LPB), and the CD14 receptor, which may be glycosylphosphatidylinositol (GPI)-anchored (mCD14) or soluble (sCD14) (3). Binding of LPS to mCD14 on myeloid cells leads to cellular activation and the production of various proinflammatory molecules, while a soluble CD14-LPS complex participates in the activation of cells that do not bear the anchored form - such as endothelial, epithelial, and smooth muscle cells (3). In contrast with what has been learned about how LPS interacts with cells, much less is known about how Gram positive bacteria stimulate cells. There is some evidence that components of Gram-positive bacteria activate monocytic cell lines by a CD14-dependant mechanism. However, CD14 independent activation of monocytes by Gram positive cell wall components has also been reported, and the integrin, L-selectin, has recently been proposed to act as a low affinity receptor for LPS (4). Regardless of the nature of infection the mediators in the progression of septic shock are believed to be the proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-8 (IL-8), along with nitric oxide (NO), produced both by cells of the immune system and by vascular endothelial cells (5-8). In recent years, intracellular signal transduction pathways that lead to cytokine production within cells of monocytic lineage have been extensively studied. This review will focus on the intracellular activation mechanisms that are initiated by LPS, and the biological consequences of cellular activation.

3. ACTIVATION OF MAP KINASE PATHWAYS.

Mitogen activated protein kinases (MAPK) are a family of proteins involved in intracellular signaling. Mammalian MAP kinases can be divided into four groups

based on their structure and function: extracellular signal-regulated kinase (ERK) (9), c-Jun N-terminal kinase or stress activated protein kinase (JNK or SAPK) (10,11), ERK5 or BMK (12,13), and p38 group (14-16). A variety of extracellular stimuli produce cellular responses via activation of the MAP kinase cascades (9,17-20). It would therefore not be surprising to find them involved in mediating LPS effects within cells. To date three MAP kinase pathways, ERK, JNK, and p38, have been reported to be activated by LPS stimulation in macrophages.

3.1 ERK

ERK is the first group of MAP kinase that was found to be activated by LPS stimulation. Using an anti-phosphotyrosine antibody, Weinstein *et al* detected rapid (4-5 min) tyrosine phosphorylation of 39-, 41-, and 44-kDa proteins within LPS stimulated macrophages (21). Two of these tyrosine phosphorylated proteins were subsequently identified as p42, (ERK2) and p44 (ERK1) (22). Activation of ERKs can occur via the upstream effector, Ras. Upon stimulation, Ras interacts with the NH_2-terminal domain of Raf-1, leading to its recruitment to the plasma-membrane, whereupon Raf-1 is phosphorylated by another kinase. Once activated, Raf-1 may phosphorylate MAP kinase kinase-1 (MKK-1, also known as MEK), which in turn, phosphorylates ERK.

What then is known about the events following the activation of MAP kinase pathways? A number of proteins have been identified as ERK substrates, including the transcription factors ELK-1 and c-Myc, cytoplasmic proteins such as cytosolic phospholipase A2 (cPLA2), and several protein kinases including p90 ribosomal S6 kinase (RSK also known as MAPKAPK1), MAP kinase activated protein kinase 3 (MAPKAPK3) (also known as 3pK) as well as the recently described MAP kinase interacting kinase 1 and 2 (MNK1/2) (9,23-25). Phosphorylation of ELK-1 and c-Myc could regulate the transcription activity of these transcription factors, however, the linkage between the activation of transcription factors by ERK and LPS induced cytokine expression has not yet been established. The downstream protein kinases of ERK should diversify ERK signal via their substrates. Phosphorylation of translational initiation factor eIF-4e by Mnk may participate in translational regulation of cytokine expression. Phosphorylation of phospholipase A2 certainly plays a role in inflammatory responses because it leads to the production arachidonic acid, which can activate non-classical PKC isoforms, and is the precursor of the eicosanoids.

On exposure to LPS, ERK-1/2 and its upstream activator, MEK-1 have been shown to be activated in monocytes, where ERK activation is absent in C3H/HeJ mice, a spontaneous mouse mutant which does not produce TNFα or IL-1β in response to LPS treatment (26). Both of these pieces of evidence indicate that ERKs may be involved in the signaling pathway that results in cytokine synthesis following LPS treatment. The Ras and Raf-1 components of the ERK cascade have been suggested to be necessary for LPS-stimulated TNFα production. This was shown from the ability of LPS to induce rapid phosphorylation of Raf-1 in macrophages, activating the MEK-1/ERK pathway. Expression of dominant negative forms of Ras and Raf-1 in macrophages also indicated their involvement in LPS-mediated cell signaling because dominant negative mutants of Ras or Raf-1 can inhibit LPS-stimulated TNFα production (26,27). However, while up-regulation of Raf-1 has been shown to strongly activate ERK1 and ERK2, it causes only a small increase in TNFα mRNA expression, and protein secretion. Thus the ERK pathway only partially mimics LPS effects, implying that other signaling pathways triggered by LPS may also play a role. Indeed, it has been demonstrated that LPS stimulation leads to the activation of multiple signal pathways, including JNK, and p38 MAP kinase pathways (14,28,29).

3.2 JNK

Both JNK1 and JNK2 have been reported to be activated in LPS treated macrophages (28,29). As their name indicates, the JNK proteins are associated with the phosphorylation of c-Jun (10,11). In addition, other transcription factors, including activating transcription factor-2 (ATF-2), and ternary complex factor (TCF) have been reported to be downstream targets for JNK (30-32). c-Jun can complex with c-fos or ATF2 which constitutes AP-1 or CRE binding activity. TCF mediated c-fos expression also influences c-Jun/c-fos dimer formation which would have impact on AP-1 binding activity. Since AP-1 and CRE sites are found in many cytokine promoters including TNF, regulation of AP-1 and/or CRE binding activity by JNK pathway plays important role in LPS induced cytokine expression. In addition, c-Jun complexes have been shown to act synergistically with NF-κB in LPS-treated monocytes to enhance the induction of TNFα (33).

Activation of JNK proteins inside cells is believed to occur through dual tyrosine and threonine phosphorylation by MKK4 (also known as SEK1, JNKK1) and MKK7 (also known as JNKK2) (34,35). MKK4 can be activated by MEKK1 (36). Thus, the sequential activation pathway may take the following route: MEKK1→MKK4/7→JNK. Events further upstream of this pathway appear to be cell type and stimuli dependent. Activation of JNK cascade by EGF or v-Src requires functional Ras. Other members of the Ras superfamily of small G proteins , Rac and Cdc42, are more effective JNK activators than Ras and can act synergistically with Ras. Once activated, Cdc42 and Rac bind and activate a protein kinase PAK1 which in turn can activate the JNK cascade (19). The upstream elements of JNK pathway in LPS stimulated macrophages have not been uncovered.

3.3 p38 MAP kinases

p38 (or p38α, also known as CSBP and RK) was initially isolated and cloned through a study designed to identify proteins in macrophages and pre-B cells that tyrosine phosphorylated in response to LPS (14,37). p38α was also cloned as a specific target of pyridinyl imidazole derivatives such as SB203580 which inhibit the production of proinflammatory cytokines by monocytes (38). Four isoforms of the p38 MAP kinases have been cloned and

characterized: p38 (p38α), p38β (39), p38gamma (also known as ERK6 or SAPK3) (40-42), and p38delta (also known as SAPK4) (43-45), each of which contains a TGY dual phosphorylation motif between domains VII and VIII - distinguishing them from the ERK kinases (TEY) and JNK kinases (TPY). p38α and p38β are sensitive to SB203580 inhibition, but the activity of p38gamma and p38delta are unaffected (43). The p38 MAP kinases are thought to have evolved from an ancestral gene and are homologous to the kinase Hog1 in budding yeast and Spc1/Sty1 in fission yeast (46-48). A *Xenopus* p38 homologue, Mpk2, has also been cloned (15). p38 isoforms are activated by MKK3 or MKK6 (34). MKK6 can in turn be phosphorylated by MEKK5, such that the following linear pathway emerges: MEKK5→MKK3/6→p38. Other upstream activators of p38 activity may include members of the Rho GTP-binding protein family, Rac and Cdc42 (49). Rac and Cdc42 GTPases have been implicated to be activators for JNK pathway (50). The activation of p38 by these small G-proteins may also through a group of serine-threonine kinases known as p21-activated kinases (Paks) (49). Expression of dominant negative Cdc42, Rac, or Pak1 resulted in abolition of p38 activity in COS-7 and HeLa cells stimulated by IL-1 or ultraviolet light (49). The picture is further complicated by the discovery of other regulators of p38 activity, including MLK3, and DAK (51), which may, or may not fit into a single linear model of p38 activation. Whether these molecules are involved in LPS induced p38 activation still need further investigation. The molecular events which succeed LPS-CD14 engagement remain to be resolved. Several proteins have been identified as substrates of p38α. These proteins include transcription factors such as CHOP10 (also known as GADD153) (52), MEF2C (53) and Sap1 in TCF (54-56), enzymes such as cPLA2 (57), and the protein kinases MAPKAPK2/3, MNK1/2 and PRAK (15,23,24,58-60). All four members of the p38 kinase family are able to phosphorylate the transcription factor activating transcription factor 2 (ATF2) *In vitro*(43). Although there is evidence that different p38 isoforms may have different functions, the activation of p38 isoforms other than p38α by LPS has not been investigated. The discovery of a specific inhibitor of p38 SB203580 has provided a useful tool for dissecting the role of p38 kinases in septic shock (61,62). Studies have demonstrated that inhibition of p38 in monocytes prevents LPS-stimulated production of IL-1β and TNF (38,63). It is clear that p38 pathways play a crucial role in LPS induced cytokine expression. However, the precise mechanism by which p38 regulates cytokine gene expression is still uncertain. Activation of MEF2C by p38 should lead to up-regulation of c-Jun expression which may influence formation of transcription complex c-Jun/c-Jun, c-Jun/c-fos and c-Jun/ATF2 (53). Thus p38 may indirectly regulate AP-1 and CRE binding activity in LPS treated macrophages. Studies using p38 specific inhibitor have demonstrated that the decrease of cytokine (such as IL-1) production resulting from p38 inhibition is an effect on translational control, because the mRNA level of IL-1 was not affected by blocking of p38α/β activity (63). A great deal of effort has been employed to study the translational regulation by p38 pathway, however, none of p38 substrates found so far have been linked to translational machinery. The downstream kinases of p38 may direct p38 effects to translational regulation, because Mnk has been shown to phosphorylate a translational initiation factor, eIF-4e at a physiological relevant phosphorylation site (23). Activation of kinases downstream of p38 may also direct p38 signal into the cytoskeleton via small heat shock protein phosphorylation. Whether these events occur in LPS treated macrophages and the role they may play in cytokine production still await further investigation.

Activation of p38 was also observed in other types of cells: p38, along with ERK1 and ERK2, has been shown to be activated in LPS-induced astrocytes, while in neutrophils, p38 is activated in the absence of ERK or JNK activation (64,65). It has been observed that exposing neutrophils to chemoattractants activates ERK and p38 kinases, and causes actin assembly, cell adherence, calcium influx, superoxide production and granule enzyme release (66). However, LPS treatment results in only p38 activity being up-regulated, and of the listed cellular responses only actin assembly and cell adherence occur (65). It is therefore possible that these may be as a result of p38 activation, since p38 has been associated with the phosphorylation of MAPKAP2, and its substrate, heat shock protein 27 (hsp27), linked to actin reorganization (67).

3.4 Coordination of MAP kinase pathways following LPS stimulation.

LPS stimulation of macrophages results in the activation of three different groups of MAP kinase: ERK, JNK, and p38. Although these kinase pathways are specifically regulated, they may integrate their function to a single target. An example of this is the regulation of ternary complex factor (TCF). Maximal c-Fos transcription is dependent on an upstream element, termed the serum response element (SRE) (68). This cis-element is constitutively bound to serum response factor (SRF) and TCF, which is composed of Elk-1, Sap-1a or Sap2. Elk-1 can be phosphorylated *In vitro* by JNK, ERK, and p38; and Sap-1a by ERK and p38 (54-56). In certain cell types such as NIH3T3 fibroblasts, both JNK and p38 are required for TCF activation, i.e. JNK and p38 function on a single target (55). The activation of c-Jun is classically associated with JNK, but p38 has also been shown to be involved in LPS-induced c-Jun expression that may influence c-Jun related transcription activities. Inhibition of MEK or p38 also has been shown to reduce stress-induced c-Jun and c-Fos transcription in NIH 3T3 and HeLa TK^- cells (56,69). Pathway synergy may additionally play a role in the expression of TNFα in response to LPS. Simultaneous activation of p38, JNK and ERK pathways by dominant active MKKs results in levels of TNFα reporter expression five fold higher than if a single pathway is active (Han *et al*, unpublished results).

4. PROTEIN KINASE C

It has been reported that PKC activation can be observed following LPS treatment, although the amplitude of activation is relatively low (2-fold). PKC refers in fact to

a family of mainly calcium- and phospholipid-dependent kinases catalyzing the phosphorylation of serine and threonine residues in proteins, and which differ in their tissue expression and reaction requirements (70). The group has been implicated in LPS mediated effects *In vivo* by Kozak *et al.* (71) who demonstrated the attenuation of LPS-induced pyrexia, and levels of circulating IL-6 in rats through the use of PKC inhibitors. *In vitro* experiments have shown that TNFα and IL-1β secretion and mRNA accumulation within monocytes following LPS treatment is dependent on PKC activity (72). Treatment of monocytes with H-7, a PKC inhibitor with tyrosine phosphorylation stimulatory effects, or with the inhibitor, staurosporin, resulted in the inhibition of TNFα and IL-1β secretion by LPS-stimulated monocytes. Since anti-CD14 antibody prevents LPS-induced TNF production without affecting PKC activation, PKC activation may be a CD14-independent pathway (73). It is clear that the PKC pathway had no effect on MAP kinase activation because activation of PKC also failed to stimulate tyrosine phosphorylation of MAP kinases (21), and PKC inhibitors have been shown to have no effect on the activity of MAPK, JNK, or p38 in stimulated macrophages. Recently, two separate groups have sought to uncover which PKC isoforms are associated with LPS treatment of monocytes. Based on earlier findings that the LPS-induced PKC activation did not require calcium or diacylglycerol (Liu *et al*, 1994), Herrera-Velit *et al.* (74) narrowed the search to PKC isoforms belonging to the atypical PKC subfamily (PKC -ζ, -λ, -ι). Fractionation of LPS-induced monocyte lysates by chromatography, and immunoblotting with anti-PKC-ζ antibodies demonstrated the presence of PKC-ζ at the peak of kinase activity. Activation of PKC-ζ is phosphatidylinositol 3-kinase (PI 3-kinase) dependent (75). Another PKC isoform activated by LPS was identified by Shapira *et al*. (76) using biochemical and immunological approaches. Like PKC-ζ, PKC-ε does not require calcium. Unlike PKC-ζ, it is activated by diacylglycerol and is tissue restricted (70,77).

5. SRC FAMILY KINASES

While the mechanism by which CD14 communicates with the cytoplasm following LPS treatment has not yet been revealed, ®tefanov< *et al* (78) described the co-precipitation of members of the Src-protein tyrosine kinase family with CD14. It was further observed that LPS treatment of monocytes resulted in increased activity of these isolated Src-kinases ($p53/56^{lyn}$, $p58/64^{hck}$ and $p59^{c\text{-}fgr}$) combined with an associated increase in tyrosine phosphorylation of the proteins. The known involvement of Src-kinases in cell signal transduction combined with these data suggested that Src-kinases may be involved in communicating a signal from CD14 to the cytoplasm. More recently however, it has been shown that transgenic mice lacking expression of $p53/56^{lyn}$, $p58/64^{hck}$ and $p59^{c\text{-}fgr}$ still produce normal levels of cytokines, phosphorylation of MAPKs and activation of NF-κB following LPS stimulation (79). These Src-kinases do not therefore seem to be necessary for LPS-stimulated cytokine expression, although this does not exclude the possibility that they are involved in other LPS-induced cellular responses, or that uncharacterized Src-kinases participate in CD14 mediated signal transduction.

6. NF-κB

Nuclear factor -κB (NF-κB) is a ubiquitous transcription factor that is up-regulated in response to various stimuli, including LPS and TNFα. Its significance in septic shock can be highlighted by its activation in tissue following LPS treatment, and by the effect of its inhibition: selective inhibition of NF-κB *in vivo*, with pyrrolietine dithiocarbamate has been shown to reduce LPS-stimulated inducible NO synthesis and to prevent systemic hypotension associated with septic shock (8). Moreover, almost all genes central to the pathogenesis of septic shock contain promoter elements for NF-κB, such as TNFα, IL-1β, IL-6, IL-8, inducible nitric oxide synthase (iNOS), and cyclo-oxygenase-2 (COX-2, which regulates prostaglandin synthesis from arachidonic acid). However, macrophages from C3H/HeJ LPS-hyporesponsive mice do not produce TNFα, but do activate normal levels of NF-κB in response to LPS, indicating that LPS-mediated cellular effects cannot be solely attributed to NF-κB alone (80).

NF-κB comprises a number proteins, p50/105, p65 (relA), p52/100, and RelB. These proteins homo- and heterodimerise to produce isoforms of NF-κB, the most abundant inducible form in mammals being p50/p65 (81). The dimer is retained in an inactive state in the cytoplasm through being sequestered by an inhibitor proteins, I-κB, which bind NF-κB, and mask the nuclear localization signal, preventing its nuclear translocation (82). Phosphorylation of I-κB at serines 32 and 36 triggers its ubiquitination leading to its subsequent degradation through the proteasome pathway, and facilitating the mobilization of NF-κB to the nucleus and regulation of responsive genes (82). The process leading to I-κB phosphorylation has recently been the subject of a great deal of study. I-κB has been found to be activated by a high molecular weight complex, known as I-κB kinase (IKK) (83,84). This complex contains two proteins with leucine-zipper and helix-turn-helix motifs (called IKK-1, and IKK-2), which can heterodimerise (85,86). Once activated by an upstream kinase, IKK phosphorylates I-κB at serines 32 and 36, which triggers its degradation. A kinase which may be responsible for activating IKK was recently discovered (87). This protein, NIK, shares homology with several MAP kinase kinase kinases, including MEKK1. NIK interacts with Traf2 - a TNF-receptor associated factor that binds to the p55 and p75 TNF receptors, and might therefore be involved with TNFα-mediated activation of NF-κB (88). On the other hand, MEKK1, the upstream activator of JNK has been shown to activate NF-κB *In vitro* and *in vivo*. This seems to occur through MEKK1 phosphorylation of the molecular weight I-κB-kinase complex, responsible for the ubiquitin-dependent phosphorylation of I-κB, prior to its degradation and NF-κB activation (82,83,89). The activation of IKK-1 and IKK-2 can be detected in LPS stimulated macrophages (V. Kravchenko, personal communication). However, the

function of MEKK1 in septic shock and the signaling elements upstream of IKK in LPS-mediated activation of NF-κB remain uncertain. Nevertheless, LPS-stimulated NF-κB activation was found to be dependent both on proteasome and tyrosine kinase activity (90,91), suggesting that phosphorylation mediated protein degradation of I-κB is used by LPS to activate NF-κB.

Despite the ability of PKC and other kinases to phosphorylate I-κB *In vitro*, specific inhibition of MAPK, PKC, PKA, cGMP-dependent protein kinase, or calmodulin-dependent protein kinase has been reported not to prevent NF-κB activation in response to LPS in a cell-free system (91). Not only does NF-κB inhibition repress the LPS-stimulated expression of COX-2 in macrophages, selective inhibitors of ERK1/2 and p38 (not JNK) cause a depression in COX-2 expression. While this suggests that NF-κB activation is not the sole regulatory element controlling the expression of these inflammatory molecules, activation of MAP cascades by hydrogen peroxide, sorbitol, or PMA did not result in COX-2 synthesis - indicating the importance of simultaneous activation of multiple pathways. Interestingly, in COS-1 cells the expression of p38 leads to the suppression of TNF-induced I-κB phosphorylation, demonstrating an interaction between MAP kinase pathways and NF-κB (92). The coordination and interaction of MAP kinase pathways and NF-κB cascade may play a key role in LPS induced gene expression.

7. MISCELLANEOUS EFFECTS OF LPS

The majority of effort has concentrated on dissecting LPS induced pathways with macrophages, since they are considered to be the primary mediators of endotoxin shock. However, endothelial cells have also been shown to react directly to endotoxin. Schumann *et al.* (93) demonstrated that endotoxin may exert a direct effect on endothelial cells through a measured increase in protein tyrosine kinase activity, ERK1 and p38, in a human umbilical vein endothelial cell line. Stimulation of the p38 cascade in endothelial cells has been shown to result in the expression of the vascular adhesion molecule 1, VCAM-1 on the cell surface (94). Expression of TNFα from leukocytes in response to LPS could also result in increased expression of both VCAM-1 (through the MAP kinase cascade) and intercellular adhesion molecule 1, ICAM-1, which can also be activated by the lipid-A component of Gram negative cell walls, though not by LPS itself (93,94). It has also been observed that LPS may activate ceramide activated protein kinase (CAK), and that this may occur through molecular mimicry. CAK is a membrane bound kinase that may directly activate ERK and JNK cascades (95). The lipid-A fraction of LPS shares regional homology with ceramide, and it therefore suggested that this may mimic the secondary messenger function of ceramide, leading to activation of MAPK pathways (96,97). LPS has also been reported to induce the myristolation of macrophage proteins, a response which is absent in C3H/HeJ mice, and may be important in LPS-mediated signaling (98).

It is also should be noticed that activation of a pathway in response to LPS leads to the secretion of molecules, such as TNF and IL-1, which then rebind cell surface receptors and elicit an autocrine response - activating other kinase pathways. C3H/HeJ mutant mice are a useful model for dissecting LPS effects within cells, because the responsiveness of this mouse to cytokines is normal. Transgenic mice deficient in the p55 TNF-receptor, one of the receptors for TNFα, are more resistant to endotoxin treatment than C3H/HeJ mice. These mice resist endotoxin effects at levels more than 1000 times higher than wild-type mice, suggesting that LPS may signal via the TNF-receptor, or (more likely) that a TNF autocrine response is involved in LPS-mediated effects.

8. CONCLUSIONS

A milieu of cellular events occur following exposure to LPS, and it is clear that a number of signaling pathways become active, including the ERK, JNK and p38 kinase pathways, and NF-κB activation pathway. These pathways lead in turn to the activation of transcription factors such as AP-1 and NF-κB which control cytokine expression. It is probable that LPS effects are mediated in part through the convergence of separate pathways onto cellular targets. LPS-stimulated effects have also been reported to be largely CD14-dependent. However, the events which immediately follow binding of LPS/LPB to CD14, and which lead to the activation of kinase cascades still remain to be resolved.

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Send correspondence to: Jiahuai Han, Dept. Immunology, The Scripps Research Unit, 10550 North Torrey Pines Road, La Jolla, CA 92037. Tel: (619)-784-8704, Fax: 619-784-8239, E-mail: jhan@scripps.edu