

CUTTING EDGE

Cutting Edge: T Cells Respond to Lipopolysaccharide Innately via TLR4 Signaling¹Alexandra Zanin-Zhorov,² Guy Tal-Lapidot,² Liora Cahalon, Michal Cohen-Sfady, Meirav Pevsner-Fischer, Ofer Lider,³ and Irun R. Cohen⁴

LPS, a molecule produced by Gram-negative bacteria, is known to activate both innate immune cells such as macrophages and adaptive immune B cells via TLR4 signaling. Although TLR4 is also expressed on T cells, LPS was observed not to affect T cell proliferation or cytokine secretion. We now report, however, that LPS can induce human T cells to adhere to fibronectin via TLR4 signaling. This response to LPS was confirmed in mouse T cells; functional TLR4 and MyD88 were required, but T cells from TLR2 knockout mice could respond to LPS. The human T cell response to LPS depended on protein kinase C signaling and involved the phosphorylation of the proline-rich tyrosine kinase (Pyk-2) and p38. LPS also up-regulated the T cell expression of suppressor of cytokine signaling 3, which led to inhibition of T cell chemotaxis toward the chemokine stromal cell-derived factor 1 α (CXCL12). Thus, LPS, through TLR4 signaling, can affect T cell behavior in inflammation. The Journal of Immunology, 2007, 179: 41–44.

Endotoxin or LPS is a Gram-negative bacterial molecule that can activate some types of host immune cells via TLR signaling (1, 2). The entry of LPS into the blood is known to be critical in Gram-negative infections, toxic shock, and, most recently, AIDS (3). Although TLR2 and TLR4 are expressed on the surface of T cells, LPS was reported not to affect T cell cytokine secretion or proliferation or to activate regulatory T cells (4–6). These negative findings led some to conclude that T cells cannot respond innately to LPS. However, T cell physiology is not limited to cytokine secretion and proliferation. Indeed, we have found that the heat shock protein HSP60 can innately regulate T cell functions such as adhesion, migration, and cytokine secretion (6–8) and that it can enhance functions of CD4⁺CD25⁺ regulatory T cells (9), all by way of TLR2 signaling.

Mindful of these aspects of T cell function, we tested whether LPS might directly affect T cell adherence or migration and can now report this to be the case. These previously unsuspected effects of LPS on T cells are likely to be important in the host-parasite interactions in Gram-negative infections and in AIDS.

Materials and Methods

Reagents

The reagents and chemicals were purchased from the sources previously described (6–8). *Salmonella minnesota* LPS purified by phenol extraction was purchased from Sigma-Aldrich. The neutralizing mAbs anti-human TLR2 and anti-human TLR4 were obtained from eBioscience.

Mice

Female C57BL/6J were obtained from Harlan Olac. C3HeB/FeJ and C3H/HeJ mice were obtained from The Jackson Laboratory. Dr. S. Akira (Osaka University, Osaka, Japan) provided TLR2^{-/-} (B6.129P2-Tlr2^{tm1Aki}) and MyD88^{-/-} (B6.129P2-MyD88^{tm1Aki}) mice (10, 11).

Human and mouse T cells

CD3⁺ T cells were purified from the peripheral blood of healthy human donors (Blood Bank, Tel-Hashomer, Israel), or from mouse spleens as previously described (6, 8).

T cell adhesion and migration assays

Analysis of T cell adhesion was determined as previously described (7). T cell chemotaxis was assayed as described using the Transwell system (8).

Western blot analysis of T cell lysates

T cells, 5 × 10⁶ per sample, were activated with different concentrations of LPS (37°C in a 7% CO₂ humidified atmosphere). Total cell lysates were prepared and analyzed for protein content as previously described (6, 7).

RNA interference

We synthesized a silent RNA (siRNA)⁵ sequence targeting suppressor of cytokine signaling (SOCS) 3 positions 80–101 relative to the start codon, 5'-AA-GAGCGAGTACCAGCTGGTG-3'; a dsRNA targeting luciferase (GL-2) was used as a control (Dharmacon Research). Transfections of freshly purified T cells were performed using the Human T cell Nucleofector kit (Amaxa Biosystems) as previously described (8). Cell migration was evaluated as described 48 h after transfection. Transfection efficiency was controlled by evaluating SOCS3 levels by Western blotting.

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⁵ Abbreviations used in this paper: siRNA, silent RNA; FN, fibronectin; Pyk-2, proline-rich tyrosine kinase 2; SDF, stromal cell-derived factor; SOCS, suppressor of cytokine signaling.

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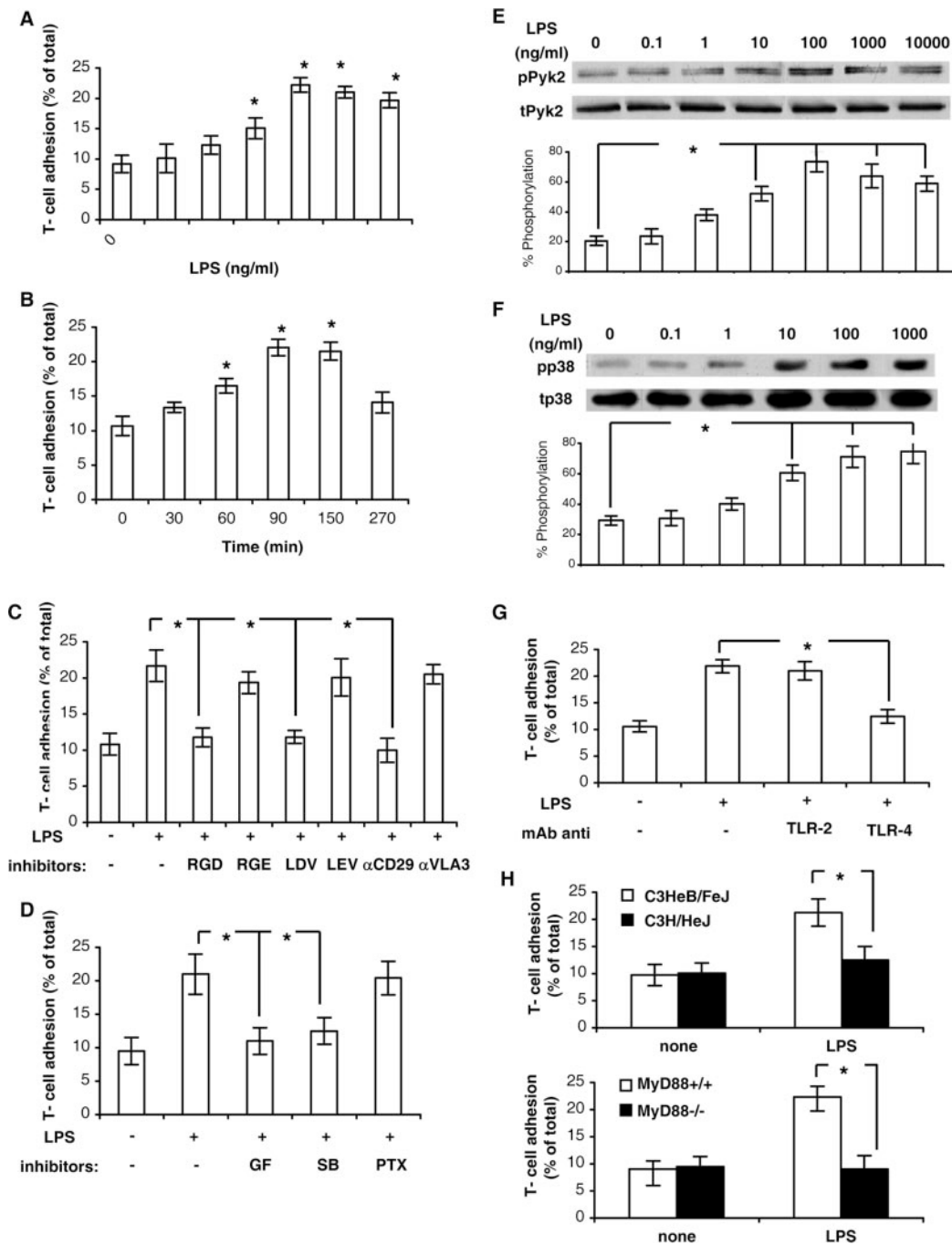


FIGURE 1. LPS induces T cell adhesion to FN via β_1 integrin and activation of PKC and p38 signaling pathways in a TLR4/MyD88-dependent manner. *A*, Purified human CD3⁺ T cells were labeled with chromium 51. The cells were then pretreated (30 min) with different concentrations of LPS and seeded onto FN-coated microtiter wells for an additional 30 min. The nonadherent cells were washed away and the remaining FN-bound cells were lysed. The radioactivity of lysates, representing the amount of FN-adherent cells, was determined. The mean \pm SD of six independent experiments using different donor bloods is shown. *B*, Time-kinetic analysis of LPS-induced T cell adhesion. T cells were exposed to 100 ng/ml LPS for different periods of time. The results are expressed as the percentage of adherent T cells. The mean \pm SD of five independent experiments is shown. *C* and *D*, T cells were pretreated with the indicated mAb (20 μ g/ml) or FN peptides (10–15 μ g/ml) or the intracellular signal transduction inhibitors GF109203X (GF; 20 nM), SB 203580 (SB; 50 nM), or pertussis toxin (PTX; 2 μ g/ml). The cells were then activated with LPS (100 ng/ml), and tested for adhesion to FN. The mean \pm SD of four different experiments is shown. *E* and *F*, T cells were exposed to LPS (100 ng/ml, 15 min) and lysed. The lysates were run on SDS-polyacrylamide gels for electrophoresis, transferred to nitrocellulose membranes, and immunoblotted with mAb anti-phospho-Pyk2 (pPyk2), anti-total Pyk2 (tPyk2), anti-phospho-p38 (pp38), and anti-total p38 (tp38). The blot of one representative experiment of five is presented. Phosphorylation levels of the experiments were estimated by densitometry, and an average percentage of phosphorylation \pm SD was calculated as (pPyk2/tPyk2) or (pp38/tp38) \times 100. *G*, T cells were pretreated with mAb anti-TLR2 or anti-TLR4 (20 μ g/ml). The cells were then treated with LPS (100 ng/ml), and percentage of adhesion to FN was determined. The mean \pm SD of four independent experiments is shown. *H*, CD3⁺ T cells purified from the spleens of wild-type C3HeB/FeJ, TLR4-mutated C3H/HeJ, MyD88^{-/-}, or MyD88^{+/+} mice were radiolabeled, pretreated with LPS (100 ng/ml) for 60 min, and seeded onto FN-coated microtiter wells. The mean \pm SD of three different experiments is shown. *, $p < 0.05$.

Statistics

Data were analyzed by Student's *t* test. Values of *p* < 0.05 was considered statistically significant.

Results and Discussion

LPS induces T cell adhesion to fibronectin via TLR4/MyD88 signaling

To assess whether LPS directly affects T cell adhesion and migration, we purified CD3⁺ T cells (> 99% purity) from the peripheral blood of healthy human donors. The purified T cells were incubated with various concentrations of LPS for various times, and we assayed the adhesion of the T cells to immobilized extracellular matrix components, namely fibronectin (FN), laminin, or type 1 collagen. We found that LPS enhanced T cell adhesion specifically to FN but not to laminin or collagen type 1 (not shown). Fig. 1*A* shows that LPS induced T cell adhesion to FN in dose-dependent manner; 100 ng/ml produced the maximal effect, and we used this standard concentration. Time-kinetic experiments revealed that treatment for 90–150 min with LPS induced peak levels of T cell adhesion to FN (Fig. 1*B*). Interestingly, longer periods (270 min) reduced T cell adherence.

APCs and B cells are very sensitive to LPS activation (1, 2). It was therefore critical to learn whether the contamination of T cells with other cell types might have been responsible for LPS-induced adhesion in our experiments. FACS analysis confirmed that our T cell preparations did not contain other cell types such as B cells, dendritic cells, or macrophages (data not shown). Moreover, incubation of purified T cells with a wide range of LPS doses (0.001–100 μg/ml) did not result in secretion of the cytokines typically secreted by APCs and B cells such as TNF-α, IL-12, IL-6, or IL-10 (data not shown). Thus, LPS can directly induce T cell adhesion to FN without involving APCs or B cells.

T cell adhesion to FN is mediated primarily by the α₄β₁ and α₅β₁ integrins (CD29) that recognize Leu-Asp-Val (LDV) and Arg-Gly-Asp (RGD), respectively. Indeed, we found that adhesion was inhibited by the RGD and LDV peptides and by anti-CD29 mAb but not by the control peptides Arg-Gly-Glu (RGE) and Leu-Glu-Val (LEV) or anti-VLA3 mAb (Fig. 1*C*).

The FN adhesion of T cells requires intracellular signaling to activate β₁ integrins (12, 13). Fig. 1*D* shows that preincubation of T cells with a protein kinase C (PKC) inhibitor, GF109203X (14), and a p38 MAPK inhibitor, SB203580 (15), but not a G protein inhibitor, pertussis toxin (16), significantly inhibited T cell adhesion induced by LPS. Moreover, a short treatment of T cells with LPS (10 min) induced the PKC-dependent phosphorylation of the focal adhesion kinase known as proline-rich tyrosine kinase 2 (Pyk-2) (14) (Fig. 1*E*) as well as phosphorylation of p38 (Fig. 1*F*). The dose-response curve was similar to that observed in the induction of adhesion: 100 ng/ml LPS induced maximal Pyk2 and p38 phosphorylation. Thus, LPS activates the phosphorylation of Pyk-2 and p38 in T cells as in monocytes and neutrophils (15, 17).

LPS activates various types of leukocytes via TLR2 and TLR4 signaling (18, 19). However, the removal of contaminants in commercial LPS preparations eliminates signaling through TLR2 (20). In this study we used LPS from *S. minnesota* purified by phenol extraction. It was recently reported that T cells express low but consistent levels of both TLR2 and TLR4 on their surfaces (5, 9, 21). Hence, we tested whether TLR2 or TLR4 are involved in the proadhesive effects of LPS on T cells. Fig. 1*G* shows that T cell adhesion to FN induced by LPS was inhibited by a neutralizing Ab to TLR4, but not by an Ab to TLR2.

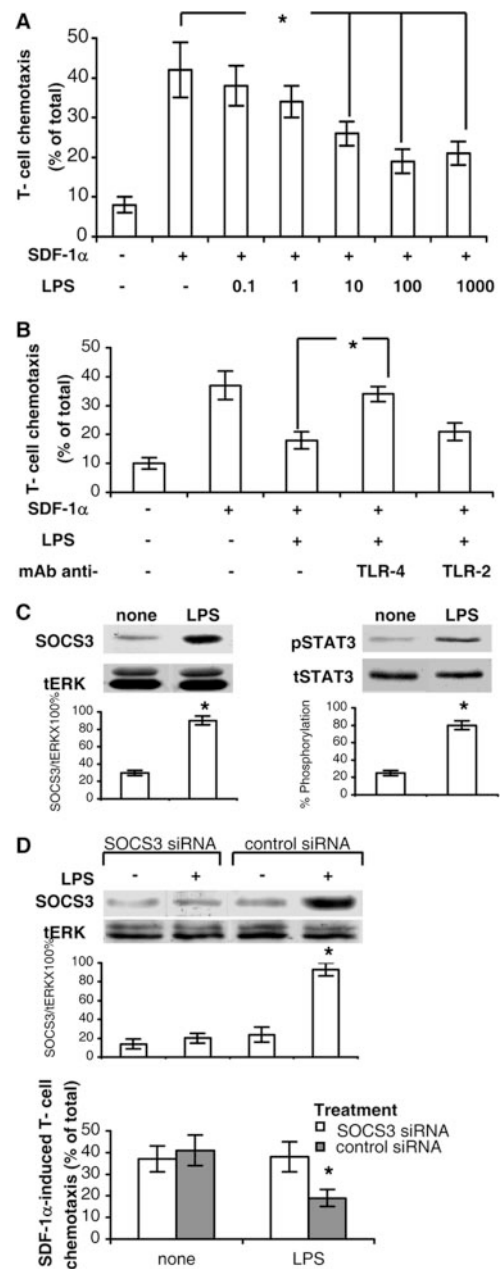


FIGURE 2. LPS inhibits T cell chemotaxis toward SDF-1α through the up-regulation of SOCS3. *A* and *B*, Human T cells were incubated with LPS (100 ng/ml) for 1 h without (*A*) or with pretreatment with mAb anti-TLR2 or TLR4 (*B*), labeled, and added to the upper chambers of Transwell apparatuses. The lower chambers contained the same media with or without SDF-1α (100 ng/ml). After 3 h, the cells that had transmigrated into the lower wells were collected, centrifuged, and lysed, and the radioactivity in the resulting supernatants was determined. The mean ± SD of five different experiments is shown. *C* and *D*, T cells without treatment (*C*) or after transfection with siRNA targeting SOCS3 or control siRNA (*D*) were incubated with LPS at 100 ng/ml for 1 h and tested for chemotaxis or lysed. Cell lysates were immunoblotted with anti-SOCS3 and anti-total ERK (tERK; evaluation of total ERK served as a control) or with anti-pSTAT3 and anti-STAT3. The levels of SOCS3, total ERK, phospho-STAT3 (pSTAT3), and total STAT3 (tSTAT3) were estimated by densitometry, and the average percentage (± SD) of the five experiments was calculated by the OD of SOCS3/tERK (pSTAT3/tSTAT3) × 100. *, *p* < 0.05.

To extend the observations to mouse T cells, we used T cells of C3HeB/FeJ (wild-type TLR4) mice, C3H/HeJ (mutated, nonfunctional TLR4) mice, TLR2 knock-out mice, or MyD88 knockout mice. Fig. 1*H* shows that LPS activated T cells from

the C3HeB/FeJ mice to adhere to FN; T cells from the C3H/HeJ mice did not respond. Moreover, T cells purified from spleens of TLR2 knockout mice responded to LPS, as did T cells from wild-type C57BL/6 mice (not shown).

LPS can signal cells both through MyD88-dependent and MyD88-independent pathways (10, 22). Fig. 1H demonstrates that MyD88-deficient T cells did not respond to LPS. Thus, T cell adhesion to FN induced by LPS requires TLR4 signaling that depends on MyD88.

LPS inhibits human T cell chemotaxis to stromal cell-derived factor (SDF)-1 α via TLR4

We incubated human T cells with various concentrations of LPS for 1 h and assayed the effect on T cell chemotaxis directed to SDF-1 α . Fig. 2A shows a dose-dependent inhibition of chemotaxis. We incubated purified CD4⁺ and CD8⁺ subsets of T cells with LPS and found no differences in the ability of LPS to inhibit their migration (data not shown). Pretreatment with a neutralizing anti-TLR4 mAb, but not an anti-TLR2 mAb (20 μ g/ml), abrogated the inhibitory effect of LPS on chemotaxis (Fig. 2B). Thus, TLR4 is also required for LPS to inhibit T cell migration.

SOCS3 mediates the inhibitory effect of LPS on human T cell chemotaxis to SDF-1 α

SOCS family proteins are negative feedback regulators of cytokine-induced JAK/STAT activation through their binding to JAK kinases (23–25). LPS can induce the expression of SOCS3 in macrophages (26). Moreover, the up-regulation of SOCS3 can inhibit CXCR4 signaling and block chemotaxis to SDF-1 α (16). Fig. 2C shows that LPS up-regulated T cell expression of SOCS3 and induced the phosphorylation of STAT3 (Fig. 2C), which is essential for SOCS3 up-regulation (23, 25).

To confirm that the inhibitory effect of LPS on chemotaxis requires SOCS3, we specifically silenced SOCS3 expression using RNA interference. This treatment abrogated the induction of SOCS3 expression by LPS; transfection with control siRNA had no effect (Fig. 2D). Moreover, the inhibitory effect of LPS on chemotaxis was also completely prevented (Fig. 2D).

The present study demonstrates that the failure of LPS to affect T cell cytokine secretion and proliferation (4, 5) does not mean that LPS has no significant effect on T cells. On the contrary, LPS can up-regulate T cell adherence to FN and down-regulate T cell chemotaxis to SDF-1 α . These effects of LPS could be important clinically; increased amounts of LPS in the blood can occur during sepsis with Gram-negative bacteria (27), and chronic HIV infection and AIDS involve markedly increased blood levels of LPS originating from gut flora (3). Hence, some of the pathological effects of LPS might be due to the direct effects of LPS on T cells. Indeed, the up-regulation of T cell adherence and the down-regulation of migration could account, at least in part, for the accumulation of activated T cells noted in the lymph nodes of HIV-infected persons (28) and possibly for other immune aberrations.

Disclosures

The authors have no financial conflict of interest.

References

- Muzio, M., N. Polentarutti, D. Bosisio, P. P. Manoj Kumar, and A. Mantovani. 2000. Toll-like receptor family and signalling pathway. *Biochem. Soc. Trans.* 28: 563–566.
- Cohen-Sfady, M., G. Nussbaum, M. Pevsner-Fischer, F. Mor, P. Carmi, A. Zanin-Zhorov, O. Lider, and I. R. Cohen. 2005. Heat shock protein 60 activates B cells via the TLR4-MyD88 pathway. *J. Immunol.* 175: 3594–3602.
- Brenchley, J. M., D. A. Price, T. W. Schacker, T. E. Asher, G. Silvestri, S. Rao, Z. Kazzaz, E. Bornstein, O. Lambotte, D. Altmann, et al. 2006. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat. Med.* 12: 1365–1371.
- Sutmoller, R. P., M. H. den Brok, M. Kramer, E. J. Bennis, L. W. Toonen, B. J. Kullberg, L. A. Joosten, S. Akira, M. G. Netea, and G. J. Adema. 2006. Toll-like receptor 2 controls expansion and function of regulatory T cells. *J. Clin. Invest.* 116: 485–494.
- Xu, D., M. Komai-Koma, and F. Y. Liew. 2005. Expression and function of Toll-like receptor on T cells. *Cell Immunol.* 233: 85–89.
- Zanin-Zhorov, A., R. Bruck, G. Tal, S. Oren, H. Aeed, R. Hershkoviz, I. R. Cohen, and O. Lider. 2005. Heat shock protein 60 inhibits Th1-mediated hepatitis model via innate regulation of Th1/Th2 transcription factors and cytokines. *J. Immunol.* 174: 3227–3236.
- Zanin-Zhorov, A., G. Nussbaum, S. Franitza, I. R. Cohen, and O. Lider. 2003. T cells respond to heat shock protein 60 via TLR2: activation of adhesion and inhibition of chemokine receptors. *FASEB J.* 17: 1567–1569.
- Zanin-Zhorov, A., G. Tal, S. Shvitiel, M. Cohen, T. Lapidot, G. Nussbaum, R. Margalit, I. R. Cohen, and O. Lider. 2005. Heat shock protein 60 activates cytokine-associated negative regulator SOCS3 in T cells: effects on signaling, chemotaxis, and inflammation. *J. Immunol.* 175: 276–285.
- Zanin-Zhorov, A., L. Cahalon, G. Tal, R. Margalit, O. Lider, and I. R. Cohen. 2006. Heat shock protein 60 enhances CD4⁺CD25⁺ regulatory T cell function via innate TLR2 signaling. *J. Clin. Invest.* 116: 2022–2032.
- Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11: 115–122.
- Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity* 11: 443–451.
- Shimizu, Y., and S. Shaw. 1991. Lymphocyte interactions with extracellular matrix. *FASEB J.* 5: 2292–2299.
- Sieg, D. J., C. R. Hauck, D. Ilic, C. K. Klingbeil, E. Schaefer, C. H. Damsky, and D. D. Schlaepfer. 2000. FAK integrates growth-factor and integrin signals to promote cell migration. *Nat. Cell Biol.* 2: 249–256.
- Zrihan-Licht, S., Y. Fu, J. Settleman, K. Schinkmann, L. Shaw, I. Keydar, S. Avraham, and H. Avraham. 2000. RAFTK/Pyk2 tyrosine kinase mediates the association of p190 RhoGAP with RasGAP and is involved in breast cancer cell invasion. *Oncogene* 19: 1318–1328.
- Dong, C., R. J. Davis, and R. A. Flavell. 2002. MAP kinases in the immune response. *Annu. Rev. Immunol.* 20: 55–72.
- Soriano, S. F., P. Hernanz-Falcon, J. M. Rodriguez-Frade, A. M. De Ana, R. Garzon, C. Carvalho-Pinto, A. J. Vila-Coro, A. Zaballos, D. Balomenos, A. C. Martinez, and M. Mellado. 2002. Functional inactivation of CXC chemokine receptor 4-mediated responses through SOCS3 up-regulation. *J. Exp. Med.* 196: 311–321.
- Ono, K., and J. Han. 2000. The p38 signal transduction pathway: activation and function. *Cell Signal.* 12: 1–13.
- Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* 395: 284–288.
- Chow, J. C., D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem.* 274: 10689–10692.
- Hirschfeld, M., Y. Ma, J. H. Wei, S. N. Vogel, and J. J. Wei. 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J. Immunol.* 165: 618–622.
- Babu, S., C. P. Blauvelt, V. Kumaraswami, and T. B. Nutman. 2006. Cutting edge: diminished T cell TLR expression and function modulates the immune response in human filarial infection. *J. Immunol.* 176: 3885–3889.
- Kaisho, T., O. Takeuchi, T. Kawai, K. Hoshino, and S. Akira. 2001. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J. Immunol.* 166: 5688–5694.
- Alexander, W. S., R. Starr, D. Metcalf, S. E. Nicholson, A. Farley, A. G. Elefanty, M. Brysha, B. T. Kile, R. Richardson, M. Baca, et al. 1999. Suppressors of cytokine signaling (SOCS): negative regulators of signal transduction. *J. Leukocyte Biol.* 66: 588–592.
- Kubo, M., T. Hanada, and A. Yoshimura. 2003. Suppressors of cytokine signaling and immunity. *Nat Immunol.* 4: 1169–1176.
- Starr, R., T. A. Willson, E. M. Vinay, L. J. Murray, J. R. Rayner, B. J. Jenkins, T. J. Gonda, W. S. Alexander, D. Metcalf, N. A. Nicola, and D. J. Hilton. 1997. A family of cytokine-inducible inhibitors of signalling. *Nature* 387: 917–921.
- Bode, J. G., A. Nimmesgern, J. Schmitz, F. Schaper, M. Schmitt, W. Frisch, D. Haussinger, P. C. Heinrich, and L. Graeve. 1999. LPS and TNF α induce SOCS3 mRNA and inhibit IL-6-induced activation of STAT3 in macrophages. *FEBS Lett.* 463: 365–370.
- Venet, C., F. Zeni, A. Viallon, A. Ross, P. Pain, P. Gery, D. Page, R. Vermesch, M. Bertrand, F. Rancon, and J. C. Bertrand. 2000. Endotoxaemia in patients with severe sepsis or septic shock. *Intensive Care Med.* 26: 538–544.
- Brenchley, J. M., T. W. Schacker, L. E. Ruff, D. A. Price, J. H. Taylor, G. J. Beilman, P. L. Nguyen, A. Khoruts, M. Larson, A. T. Haase, and D. C. Douek. 2004. CD4⁺ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J. Exp. Med.* 200: 749–759.