**IRAK1 Serves as a Novel Regulator Essential for Lipopolysaccharide-induced Interleukin-10 Gene Expression**

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Being one of the key kinases downstream of Toll-like receptors, IRAK1 has initially thought to be responsible for NFκB activation. Yet IRAK1 knock-out mice still exhibit NFκB activation upon lipopolysaccharide (LPS) challenge, suggesting that IRAK1 may play other uncharacterized function. In this report, we show that IRAK1 is mainly involved in Stat3 activation and subsequent interleukin-10 (IL-10) gene expression. Splenocytes from IRAK1-deficient mice fail to exhibit LPS-induced Stat3 serine phosphorylation and IL-10 gene expression yet still maintain normal IL-1β gene expression upon LPS challenge. Mechanistically, we observe that IRAK1 modification upon LPS challenge leads to its modification, nuclear distribution, and interaction with Stat3. IRAK1 can directly use Stat3 as a substrate and cause Stat3 serine 727 phosphorylation. In addition, nuclear IRAK1 binds directly with IL-10 promoter in vitro upon LPS treatment. Atherosclerosis patients usually have elevated serum IL-10 levels. We document here that IRAK1 is constitutively modified and localized in the nucleus in the peripheral blood mononuclear cells from atherosclerosis patients. These observations reveal the mechanism for the novel role of IRAK1 in the complex Toll-like receptor signaling network and indicate that IRAK1 regulation may be intimately linked with the pathogenesis and/or resolution of atherosclerosis.

Innate immunity signaling mediated by Toll-like receptors (TLRs) leads to the expression of a wide variety of genes (1–4). Numerous transcription factors including nuclear factor-κB (NFκB), activator protein-1 (AP-1), interferon regulatory factors, and signal transducers and activators of transcription (Stats) are shown to be activated upon challenges with various TLR ligands (4). The mechanism for the selective activation of distinctive transcription factor is not clearly understood and may be caused by the differential recruitment of intracellular adaptor molecules such as MyD88, Mal/TIRAP, TRIF, and TRAM as well as the downstream IRAK kinases (4).

IRAK1 was the first IRAK family kinase being identified to be associated with the intracellular domain of IL-1 receptor (5). Since TLRs share the TIR domain with IL-1 receptor, it was hypothesized that IRAK1 may also participate in TLR mediated signaling. Subsequent work (6–9) including ours has confirmed that indeed various TLR ligands can activate endogenous IRAK1 kinase activation. Biochemically, we and others (7, 10, 11) have shown that IRAK1 undergoes covalent modification likely due to phosphorylation and ubiquitination upon IL-1 or LPS challenge. IRAK1 can form a complex with MyD88, as well as TRAF6 (6). Since the most apparent downstream target of LPS signaling is the activation of NFκB, IRAK1 is the apparent candidate to fulfill such role. Therefore, IRAK1 has historically been linked with IL-1/LPS-mediated NFκB activation. Yet the majority of published evidence supporting the role of IRAK1 in mediating IL-1/LPS-induced NFκB activation has been derived from studies employing cell lines with IRAK1 overexpression (12–14). Upon overexpression, both the wild type and the kinase-dead IRAK1 (which has a point mutation in the ATP-binding pocket (K239S)) or in the catalytic site (D340N)) can strongly induce NFκB reporter activation (12, 14). The fact that despite being an active kinase, its kinase activity is not required for its function raises the concern that IRAK1 may perform other novel unidentified function besides activating NFκB.

Three related IRAK genes have been later identified, namely IRAK2, IRAK-M, and IRAK4 (13, 15, 16). All IRAKs consist of a conserved N-terminal death domain and a central kinase domain. Upon overexpression, each of these IRAKs can activate the NFκB reporter gene, suggesting that they may play redundant roles in activating NFκB (13, 17). However, studies using transgenic mice have indicated otherwise. So far, IRAK1−/−, IRAK4−/−, and IRAK-M−/− transgenic mice have been generated. Mice with IRAK4 disruption exhibit marked reduction in NFκB activation upon LPS challenge (18). Furthermore, sequence comparison with the fly IRAK counterpart pelle kinase suggests that IRAK4 is the structural orthologue of fly pelle kinase (16). These studies and analyses indicate that IRAK4 is the default kinase responsible for activating NFκB. In contrast, deletion of IRAK-M was shown to lead to elevated NFκB activity and pro-inflammatory gene expression such as tumor necrosis factor α, indicating that IRAK-M may negatively regulate NFκB activation (19). Intriguingly, IRAK1-deficient mice still retain LPS-induced NFκB activation (20), suggesting that IRAK1 may rather fulfill other distinct yet unidentified function in LPS/LTR signaling. Besides NFκB activation, TLR ligands such as LPS can also activate other transcription factors such as interferon regulatory factors and Stats (21, 22). Whether and which particular IRAK participates in the activation of interferon regulatory factors and/or Stats is not clear.

In this study, we have characterized the gene expression pattern of murine splenocytes from wild type and IRAK1-deficient mice. We have found that IL-10 induction is severely
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compromised in IRAK1-deficient cells upon LPS challenge. In contrast, IL-1β gene expression is induced to a similar extent. Since Stat3 has been shown to be critical for IL-10 gene expression, we have further studied the Stat3 activation and phosphorylation status. We have observed that IRAK1-deficient cells exhibit defective nuclear Stat3 serine 727 phosphorylation. Furthermore, we have shown that LPS induces IRAK1 modification and nuclear localization. In addition, nuclear IRAK1 interacts with Stat3 as well as endogenous IL-10 promoter element upon LPS treatment.

IL-10 is undetectable in normal healthy human sera or blood cells. However, under many pathological circumstances such as atherosclerosis, IL-10 message and protein can be readily detectable in the sera (23). The production of IL-10 may help alleviate excessive inflammation and therefore be beneficial for the resolution of atherosclerosis. We have examined the IRAK1 status in the peripheral blood mononuclear cells (PBMC) obtained from healthy and atherosclerosis patients. Our study shows that IRAK1 is constitutively modified and localizes inside the nucleus in atherosclerosis patient blood mononuclear cells (PBMC). Our finding reveals a novel role of IRAK1 in specifically mediating LPS-induced Stat3 activation and IL-10 expression.

MATERIALS AND METHODS

**Reagents**—Escherichia coli 0111:B4 LPS was obtained from Sigma. Antibody against IRAK1 was purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies against Stat3, phospho-Stat3(Ser727), and phospho-Stat3(Tyr705) were from Cell Signaling (Beverly, MA). Murine and Biosystems).

**Expression**—Vitro Transcription/Translation and Phosphorylation Assay of Stat3—Wild type IRAK1 and wild type and Stat3-S727A mutant proteins were synthesized using the pflag-IRAK1, wild type Stat3, and Stat3-S727A mutant plasmids with the Promega TNT quick-coupled transcription/translation system (Promega). In vitro synthesized IRAK1 was incubated with either Stat3 or Stat3-S727A protein at 37 °C for 30 min in 50 mM HEPES, pH 7.9, 1 mM MgCl2, 0.5 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin). After centrifugation for 3 min at 12,000 rpm, the supernatant cytoplasmic fractions were transferred and saved. Pellets containing intact nuclei were lysed and solubilized with the high salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.4 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) for 30 min and yielded the nuclear extracts. Immunoprecipitation and Western detection of corresponding proteins were performed as described (7).

**Chromatin Immunoprecipitation Assay**—Fresh and LPS-stimulated cells were fixed by adding formaldehyde (HCHO, from a 37% HCHO, 10% methanol stock (Calbiochem) into the medium for a final formaldehyde concentration of 1% and incubated at room temperature for 10 minutes with gentle shaking. Incubation with the lysis buffer was extended to 20 min at 4 °C. The chromatin was sheared by sonication using a Branson 250 sonicator with microtip at a power setting of 2 and 40% duty cycle. The samples were placed on ice for 1 min between sonication bursts. Each condition was divided into two samples, providing a pre-immunoprecipitation or “input” sample that was not incubated with specific antibodies, and an immunoprecipitated “IP” sample that was incubated overnight with antibodies specific for IRAK1 (Upstate Biotechnology, Lake Placid, NY). Preimmune IgG was used as a negative control. DNA was isolated by phenol/chloroform extraction, ethanol precipitated, and re-suspended in 20 μl of distilled H$_2$O. 4 μl of immunoprecipitated DNA was used for each PCR. The following primers specific for the IL-10 promoter −3000 to −60 bp region were used: 5’ CAG CTG TTC TTC CCA GGA AA 3’ and 5’ AGG GAG GCC TTC TCA ATG T 3’. PCR products were separated on a 2% agarose gel. The amplified band was visualized using Bio-Rad Gel Doc™

**Data Analysis**—The significance of the data was evaluated by means of one-factor analysis of variance followed by Student-Newman-Keuls test using SPSS 10.0 software. A p value <0.05 was considered statistically significant.

**RESULTS**

**IRAK1 Is Critical for IL-10 Gene Expression**—To identify potential downstream gene targets of IRAK1, we prepared total RNAs from wild type and IRAK1-deficient spleenocytes treated with or without LPS. Isolated RNAs were then used to perform cDNA microarray analysis using the Affymetrix mouse chip U74v2 and the data analyzed using the Affymetrix 4 and gene-spring data analysis software. We observed that several typical pro-inflammatory genes under the control of NFκB such as IL-1β and tumor necrosis factor α were induced to similar levels by LPS in both wild type and IRAK1-deficient spleenocytes. In contrast, we identified that IL-10 message was only induced by LPS in wild type, but not IRAK1-deficient, spleenocytes.

To confirm the microarray data, we performed real-time PCR analysis of the induced IL-10 as well as IL-1β messages. Total RNAs isolated from wild type and IRAK1-deficient mice with or without LPS treatment were reverse-transcribed into cDNAs and subsequently subjected to real-time PCR analysis using the assay-on-demand IL-10, IL-1β, and the control GAPDH primer sets purchased from Applied Biosystems. GAPDH message levels remain steady in all the samples assayed. IL-10 message levels were undetectable after 40 cycles of amplifications in resting spleenocytes from wild type as well as IRAK1-deficient mice. Upon LPS challenge, there was significant induction of IL-10 message in the wild type spleenocytes (Fig. 1a). Based on the comparative Ct method, the induction of IL-10 by LPS was greater than at least 30-fold. In contrast, there was no statistically significant difference between IL-10 message lev-
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Fig. 1. Selective suppression of IL-10, not IL-1β, expression in IRAK1-deficient splenocytes. a, LPS only induced IL-10 mRNA expression in splenocytes from wild type but not IRAK1-deficient mice. Primary splenocytes were either left untreated or treated with LPS for 4 h. Cells were collected to examine the expression of IL-10 as well as GAPDH mRNAs by real-time PCR. b, IL-1β mRNA was induced to the similar levels by LPS in both wild type and IRAK1-deficient splenocytes. Primary splenocytes were treated as described for a. The expression of IL-1β and GAPDH mRNAs was analyzed. c, the amplified IL-10, IL-1β, and GAPDH mRNAs were visualized on agarose gel. d, LPS-induced IL-10 protein production from splenocytes was suppressed in IRAK1-deficient mice. Splenocytes from wild type and IRAK1-deficient mice were treated with LPS for 16 h or left untreated. Supernatants were collected and IL-10 protein levels were quantified by ELISA. *, p < 0.05 versus wild type; #, p < 0.05 versus IRAK1−/−. All points represent the mean and S.D. from three different experiments. WT, wild type.

IL-10 reporter gene activation in cells co-transfected with wild type Stat3 construct. In contrast, there was no induction of IL-10 reporter activity in cells co-transfected with the Stat3 S727A or Y705F mutant, confirming that serine 727 as well as tyrosine 705 phosphorylation is essential for Stat3-mediated IL-10 gene transcription.

We then examined the Stat3 phosphorylation status in wild type and IRAK1-deficient splenocytes. Splenocytes from wild type and IRAK1-deficient mice were treated with LPS. Total protein extracts were harvested and separated on SDS-PAGE. Total Stat3 as well as phosphorylated Stat3 were monitored by Western blot using anti-Stat3 and anti-phospho-Stat3 antibodies. As shown in Fig. 3, LPS induced Stat3 phosphorylation at both serine 727 and tyrosine 705 residues in wild type splenocytes. In contrast, LPS only induced Stat3 tyrosine phosphorylation, but failed to induce serine 727 phosphorylation, in IRAK1-deficient splenocytes (Fig. 3a). We further fractionated the cell extracts into cytoplasmic and nuclear fractions. Total Stat3 was present in both the cytoplasmic and nuclear fractions of splenocytes from wild type and IRAK1-deficient mice (Fig. 3b). LPS induced a dramatic increase of nuclear Stat3 serine 727 phosphorylation in the wild type splenocytes. Strikingly, serine 727-phosphorylated Stat3 was completely absent in the nuclear fraction from IRAK1-deficient splenocytes (Fig. 3b).

We also examined whether IRAK1 could directly phosphorylate Stat3 serine 727. Wild type Stat3, Stat3-S727A mutant, as well as IRAK1 proteins were synthesized via in vitro transcription/translation as described under “Materials and Methods.” In vitro kinase assays were performed by mixing IRAK1 protein with either wild type Stat3 or Stat3-S727A mutant protein in the kinase buffer at 37 °C. Reaction products were resolved by SDS-PAGE. Total and Ser727-phosphorylated Stat3 proteins were detected through Western blot using anti-Stat3 and anti-Stat3-Ser727 antibodies. As shown in Fig. 3c, IRAK1 can directly cause Stat3 serine 727 phosphorylation.

IRAK1 and Stat3 Form a Novel Complex in the Nucleus—IRAK1 is known to undergo modification such as phosphorylation and ubiquitination upon IL-1 and/or LPS challenge. Intriguingly, it has been noted that IL-1β treatment may induce IRAK1 to localize inside the nucleus (30). To further determine the molecular mechanism for IRAK-mediated Stat3 activation, we analyzed IRAK1 protein status upon LPS challenge. Wild type splenocytes treated with or without LPS were harvested and used to prepare whole cell extract, cytoplasmic extract, as
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Phosphorylation as reported by others (12). We then examined the subcellular distribution of IRAK1 upon LPS challenge. Strikingly, as shown in Fig. 4b, unmodified IRAK1 (~85 kDa) was only detectable in the cytoplasmic extract and completely absent in the nuclear extract. In contrast, modified IRAK1 was primarily present in the nuclear extract (Fig. 4b).

We next asked whether IRAK1 could form a complex with endogenous Stat3. Immunoprecipitated IRAK1 complex from the cytoplasmic as well as nuclear extracts were separated on SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with anti-Stat3 antibody. As shown in Fig. 4c, Stat3 was only detected in the IRAK1 immunoprecipitated complex obtained from the nuclear extract. Furthermore, the intensity of co-immunoprecipitated Stat3 in the nucleus increased dramatically from the samples treated with LPS. We further performed immunoprecipitation of Stat3 using the cytoplasmic and nuclear extracts. As shown in Fig. 4c, Stat3 existed in both the nuclear and cytoplasmic fractions. The total Stat3 levels remained steady in resting as well as LPS treated splenocytes. Consistently, the modified IRAK1 (100 kDa) was present in the immunoprecipitated Stat3 complex in the nuclear fraction upon LPS treatment (Fig. 4c).
Modified IRAK1 Resides in the Nuclear Fraction of the Human Peripheral Blood Mononuclear Cells as Well as the Monocytic THP-1 Cells—We subsequently examined the IRAK1 status in human primary blood mononuclear cells (PBMC) as well as the THP-1 cells. As shown in Fig. 5, IRAK1 underwent modification upon LPS treatment in THP-1 cells (Fig. 5a) as well as in human primary PBMC (Fig. 5b). Furthermore, modified IRAK1 primarily localized in the nuclear fraction. Similar to the finding observed in mice splenocytes, modified IRAK1 formed a complex with the endogenous Stat3 in the nucleus upon LPS challenge (Fig. 5, a and b).

IRAK1 Binds Directly to the Endogenous IL-10 Promoter Region—Since IRAK1 is distributed into the nucleus and interacts with Stat3 upon LPS challenge, and its presence is critical for IL-10 gene expression, we hypothesized and tested to see whether IRAK1 may interact with the IL-10 promoter element. The IL-10 promoter region responsive to LPS challenge has been previously mapped to the −300- to −60-bp region (22). We therefore performed chromatin immunoprecipitation assays (Chips) to test whether IRAK1 can bind with this region in vivo. THP-1 cell treated with and without LPS were fixed with 1% formaldehyde for 10 min and subjected to Chip assays as described under “Materials and Methods.” As shown in Fig. 6, there was no binding of IRAK1 to the IL-10 promoter element in the resting cells. Strikingly, LPS induced binding of IRAK1 to the endogenous IL-10 promoter region based on Chip assay.

Blood Cells from Atherosclerosis Patients Have Elevated IRAK1 Modification and Constitutive IL-10 Expression—Inflammation has been well noticed to be intimately linked with the pathogenesis of atherosclerosis (31). Elevated IL-10 levels have been detected in human sera from atherosclerosis patients (23). The presence of IL-10 may be a self-protective mechanism to prevent excessive inflammation. The mechanism for elevated IL-10 expression in human atherosclerosis patients is not clear. We indeed found that the sera from 11 atherosclerosis patients all exhibited detectable IL-10 protein based on ELISA (~5 pg/ml). In contrast, there was no detectable IL-10 in healthy human sera. We further isolated PBMC from healthy as well as patient blood. The expression levels of IL-10 protein were determined by ELISA. As shown in Fig. 7a, PBMC from patients constitutively secreted IL-10 protein. Based on our finding that IRAK1 plays a critical role in the induction of IL-10, we therefore compared the IRAK1 status in the PBMC from healthy donors and atherosclerosis patients. As shown in Fig. 7b, IRAK1 primarily exists as the modified 100-kDa form in the patient PBMC. We further obtained the cytoplasmic and nuclear extracts from patient PBMC and analyzed the distribution of IRAK1. IRAK1 protein was immunoprecipitated from the prepared extracts and separated on SDS-PAGE. The presence of IRAK1 was subsequently monitored through Western blot using anti-IRAK1 antibody. As shown in Fig. 7c, the modified IRAK1 protein was constitutively migrated as the modified 100-kDa form and resided exclusively in the nuclear fraction from the patient PBMC.

**DISCUSSION**

Our current study has presented evidence indicating that IRAK1 plays a novel and critical role in Stat3-mediated IL-10 gene expression. IRAK1 is essential for Stat3 serine 727 phosphorylation. IRAK1 forms a complex with Stat3 as well as the IL-10 promoter element in the nucleus upon LPS challenge. Furthermore, atherosclerosis patients exhibit constitutive nuclear distribution of modified IRAK1 in their peripheral blood mononuclear cells, in concert with constitutively elevated blood IL-10 levels. This report has revealed some novel biochemical aspects of IRAK1 protein regulation. It is known that IRAK1 undergoes covalent modification such as phosphorylation and ubiquitination upon IL-1 and/or LPS challenge (7, 10, 12). Its modification may eventually lead to its subsequent degradation (11). Using human THP-1 cells, primary blood mononuclear cells, as well as mice splenocytes, we have confirmed numerous previous studies that there are indeed two signature forms of IRAK1, one being the unmodified 85-kDa form and the other being the modified (phosphorylated and/or ubiquitinated) 100-kDa form. Consistent with previous studies, upon LPS challenge, we have observed that the level of the modified IRAK1 form increases, while the unmodified form decreases. With regard to its subcellular distribution, there is only one published report show-
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Functionally, our findings serve to clarify the elusive role of IRAK1 in LPS-mediated innate immunity signaling. As described in the Introduction, although overexpression of IRAK1 may lead to NFκB reporter activation, studies using IRAK1-deficient mice have indicated that LPS can still induce NFκB activation in IRAK1-deficient cells (20). Therefore, IRAK1 may perform other unidentified roles besides NFκB activation. Our data presented herein unveil that IRAK1 is critically involved in the nuclear Stat3 serine phosphorylation and subsequent IL-10 gene expression. It has been documented that Stat3 is responsible for increased IL-10 gene expression upon LPS challenge (22). LPS can induce Stat3 phosphorylation at both serine 727 and tyrosine 705 residues (22), and Stat3 phosphorylation at both Tyr705 and Ser727 is critical for its maximum transcriptional activity (29). Our observation herein concurs with these studies. Overexpression of Stat3 with serine 727 to alanine mutation or tyrosine 705 to phenylalanine mutation fails to mediate LPS-induced IL-10 gene reporter activity (Fig. 2). Janus kinase 3 (JAK3) has been shown to be the main kinase responsible for Stat3 tyrosine phosphorylation (33). To date, the mechanism for LPS-induced Stat3 serine 727 phosphorylation is not clear. Consistent with previous reports, we have documented that LPS can induce Stat3 Ser727 and Tyr705 phosphorylation in wild type murine splenocytes. Strikingly, we have observed that although LPS-induced Tyr727 phosphorylation is normal, LPS-induced Stat3 Ser727 phosphorylation is greatly compromised in IRAK1-deficient splenocytes (Fig. 3). Furthermore, we have observed that nuclear Stat3 serine phosphorylation is completely absent in IRAK1-deficient splenocytes. In contrast, there is an increase in nuclear Stat3 serine phosphorylation in wild type splenocytes upon LPS challenge. The increased Stat3 serine phosphorylation inside the nucleus upon LPS challenge correlates well with our observation that LPS facilitates IRAK1 and Stat3 interaction inside the nucleus. We have documented in this report such interaction using mice splenocytes, human THP-1 cells, as well as human peripheral blood mononuclear cells. Furthermore, our in vitro analyses indicate that IRAK1 can directly use Stat3 as its substrate and induce Stat3 serine 727 phosphorylation. This is the first evidence revealing the biological substrate of IRAK1. Taken together, our data indicate that IRAK1 is essential for Stat3 serine phosphorylation inside the nucleus.

Although our study agrees with others that Stat3 phosphorylation is critical for IL-10 gene expression, there is another novel aspect of our present finding. It was thought that upon stimulation with various ligands, Stat3 is phosphorylated and then enters the nucleus (34). Our data consistently indicate that Stat3 is constitutively localized in the cytoplasm as well as the nucleus. LPS stimulation induced a dramatic increase of nuclear Stat3 serine 727 phosphorylation in the wild type splenocytes without affecting the total Stat3 protein levels in the cytoplasm and nucleus. Therefore, the function of Stat3 serine phosphorylation may not be involved in the Stat3 nuclear entry but rather is critical for LPS-induced IL-10 transcriptional regulation inside the nucleus.

Besides activating Stat3, which is critical for IL-10 gene expression, our study also reveals the intriguing phenomenon that IRAK1 may directly serve as a transcriptional regulator for IL-10 gene transcription. Using the Chip assay, our present study presents compelling evidence showing that endogenous nuclear IRAK1 can specifically bind with the IL-10 promoter element in vivo upon LPS challenge (Fig. 6). Further detailed studies are warranted to decipher the mechanism for IRAK1 phosphorylation and/or ubiquitination may be critical for its trafficking into the nucleus and its subsequent function in activating IL-10 gene expression. Indeed increasing evidence has shown that protein modification such as ubiquitination and/or sumoylation are critical for subcellular trafficking as well as signal transduction besides proteasome-mediated degradation (32). Further biochemical work is needed to elucidate the exact mechanism for LPS-induced IRAK1 modification and its subsequent function.
binding with the IL-10 promoter element and subsequent regulation of IL-10 gene expression.

IL-10 expression is completely absent in healthy human blood cells. However, it has long been noticed that IL-10 levels are elevated in the blood sera of atherosclerosis patients. Elevated IL-10 levels may be a self-protective mechanism preventing excessive inflammation and limiting the progression of atherosclerosis. The mechanism for the increased IL-10 gene expression is not clear. Our data indicate that IRAK1 protein in the atherosclerosis patient blood samples is constitutively modified and localized in the nucleus. Elevated IRAK1 modification and nuclear localization may lead to elevated IL-10 gene expression.

Taken together, the data presented in this study provide a novel role for the IRAK1 molecule in activating Stat3 and nuclear localization may be intimately linked with either the progression or resolution of atherosclerosis.

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REFERENCES
1. Vogel, S. N., Fitzgerald, K. A., and Fenton, M. J. (2003) Mol. Interv. 3, 466–477
2. Akira, S. (2001) Adv. Immunol. 78, 1–56
3. Aderem, A. (2001) Crit. Care Med. 29, S16–S18
4. Beutler, B., Hoebe, K., Du, X., and Ulevitch, R. J. (2003) J. Leukocyte Biol. 74, 479–485
5. Cao, Z., Henzel, W. J., and Gao, X. (1996) Science 271, 1128–1131
6. Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C. A., Jr. (1998) Mol. Cell 2, 253–259
7. Li, L., Cousart, S., Hu, J., and McCall, C. E. (2000) J. Biol. Chem. 275, 23340–23345
8. Moors, M. A., Li, L., and Mizel, S. B. (2001) Infect. Immun. 69, 4424–4429
9. Jacinto, R., Hartung, T., McCall, C., and Li, L. (2002) J. Immunol. 168, 6136–6141
10. Yamin, T. T., and Miller, D. K. (1997) J. Biol. Chem. 272, 21540–21547
11. Li, X., Commene, M., Jiang, Z., and Stark, G. R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4461–4465
12. Li, X., Commene, M., Burns, C., Vithalani, K., Cao, Z., and Stark, G. R. (1999) Mol. Cell Biol. 19, 4643–4652
13. Wesche, H., Gao, X., Li, X., Kirschnning, C. J., Stark, G. R., and Cao, Z. (1999) J. Biol. Chem. 274, 19403–19410
14. Maschera, B., Ray, K., Burns, K., and Volpe, F. (1999) Biochem. J. 339, 227–231
15. Muzzio, M., Ni, J., Feng, P., and Dixit, V. M. (1997) Science 278, 1612–1615
16. Li, S., Strelev, A., Fontana, E. J., and Wesche, H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5567–5572
17. Qin, J., Jiang, Z., Qian, Y., Casanova, J. L., and Li, X. (2004) J. Biol. Chem. 279, 26748–26753
18. Suzuki, N., Sunuki, S., Duncan, G. S., Millar, D. G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S., Penninger, J. M., Wesche, H., Ohashi, P. S., Mak, T. W., and Yeh, W. C. (2002) Nature 416, 750–756
19. Kohayashi, K., Hernandez, I. D., Galan, J. E., Janeway, C. A., Jr., Medzhitov, R., and Flavell, R. A. (2002) Cell 110, 191–202
20. Swantek, J. L., Toon, M. F., Cobb, M. H., and Thomas, J. A. (2000) J. Immunol. 164, 4301–4306
21. Fitzgerald, K. A., Rowe, D. C., Barnes, B. J., Caffrey, D. R., Visintin, A., Latz, E., Monks, B., Pitha, P. M., and Golenbock, D. T. (2003) J. Exp. Med. 198, 1043–1055
22. Benkhart, E. M., Siedlar, M., Wedel, A., Werner, T., and Ziegler-Heitbrock, H. W. (2000) J. Immunol. 165, 1612–1617
23. Mallat, Z., Heymes, C., Ohan, J., Faggion, E., Lesche, G., and Tedgui, A. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 611–616
24. Feldmann, K., Sebald, W., and Knaus, P. (2002) Eur. J. Immunol. 32, 1393–1402
25. Mueller, L. P., Yota, B. K., Neuhaus, K., Loeser, C. S., Cousart, S., Chang, M. C., Meredith, J. W., Li, L., and McCall, C. E. (2003) Shock 16, 430–437
26. Li, T., Hu, J., and Li, L. (2004) Mol. Immunol. 41, 85–92
27. Re, F., and Strominger, J. L. (2002) J. Biol. Chem. 277, 23427–23432
28. Gomez-Angelats, M., and Cidlowski, J. A. (2003) Cell Death Differ. 10, 791–797
29. Wen, Z., Zhong, Z., and Darnell, J. E., Jr. (1995) Cell 82, 241–250
30. Bol, G., Kreuzer, O. J., and Brigelius-Flohe, R. (2000) FEBS Lett. 477, 73–78
31. Ross, R. (1999) Am. Heart J. 138, S419–S420
32. Pickart, C. M. (2001) Mol. Cell 8, 499–504
33. Levy, D. E., and Darnell, J. E., Jr. (2002) Nat. Rev. Mol. Cell. Biol. 3, 651–662
34. Schindler, C., Shuai, K., Prezioso, V. R., and Darnell, J. E., Jr. (1992) Science 257, 899–813