The binding of immune complexes to macrophage Fcγ receptor results in a subsequent inhibition of lipopolysaccharide-stimulated interleukin-12 synthesis without affecting the induction of tumor necrosis factor-α. RNA interference targeting MAST205, a 205-kDa serine/threonine kinase, and transfection of dominant negative MAST205 mutants also mimic this type II macrophage phenotype. Our previous epistasis experiments suggested that the position of MAST205 in the TLR4 signal pathway was proximal to the IκB kinase complex. We now report that MAST205 forms a complex with TRAF6, resulting in the inhibition of TRAF6 NF-κB activation. We have identified a peptide (residues 218–233) from the N terminus of MAST205 that, when coupled to a protein transduction domain, inhibits the lipopolysaccharide-stimulated activation of NF-κB, modulates the size of the MAST205/TRAF6 complex, and inhibits ubiquitination of TRAF6. A dominant negative N-terminal MAST205 deletion mutant also inhibits TRAF6 ubiquitination. The domain required for degradation of MAST205 after Fcγ receptor activation resides within the N-terminal 261 residues, and degradation is triggered by protein kinase C isoform phosphorylation of Ser/Thr residues. These results suggest that MAST205 functions as a scaffolding protein controlling TRAF6 activity and, therefore, plays an important role in regulating inflammatory responses.

MAST205 is a 205-kDa Ser/Thr kinase that is widely expressed at low levels and highly expressed in spermatids, where MAST205 associates with microtubules. At neuromuscular synapses MAST205 interacts with β-2 syntrophin (1, 2). In addition to a well conserved protein kinase C/A kinase domain, MAST205 has a PDZ domain, a protein-protein recognition module (3). The role of MAST205 in cellular physiology, however, has yet to be determined. We have found that MAST205 plays a central role in the regulation of LPS-induced cytokine responses of macrophages (4). In this paper we analyze in greater detail the position of MAST205 in LPS-induced signal pathways and the factors regulating stability of the protein.

Macrophages respond to LPS by secreting proinflammatory cytokines such as tumor necrosis factor-α, IL-1, IL-6, IL-8, and IL-12. IL-12, in particular, is required for the induction of a T helper 1 response that facilitates the clearance of intracellular pathogens. The signal cascade leading to the inflammatory response is complex, reflecting both the importance of controlling the synthesis of potentially harmful cytokines and metabolites, such as reactive oxygen intermediates, and the need to tailor the response depending on the pathogen (5). The inflammatory response is also influenced by previous physiological stimuli to the responding cells, which has lent credence to the concept of macrophage subsets. For example, LPS-stimulated synthesis of IL-12 by macrophages, which is required for the induction of interferon-γ from NK cells (6), is inhibited by immune complex activation of FcγR. As a result, such type II macrophages (7) as antigen presenting cells drive the synthesis of IL-4 by T cells and murine IgG1 by B cells, characteristics of a T helper 2 response.

TRAF6, an E3 ubiquitin ligase, plays a pivotal role in the activation of downstream kinases in the IKK complex. TRAF6 catalyzes the formation of Lys-63-coupled polyubiquitin chains, and a ubiquitin K63R mutant inhibits signaling by the IL-1 receptor, which shares many signaling components with TLR4, including TRAF6 (8). The detailed mechanism by which Lys-63 polyubiquitin chains activate downstream components is not clear nor is the mechanism by which upstream interleukin-1 receptor-associated kinase kinases control TRAF6. Therefore, characterization of novel TRAF6-interacting proteins may shed light on these critical events in inflammatory pathways.

Dominant negative MAST205 mutants and RNAi constructs targeting MAST205 inhibit LPS-induced IL-12 p40 synthesis and NF-κB activation (4), showing that MAST205 is involved in the modulation of the innate immune response. Previous epistasis experiments suggested that MAST205 acted before the IKK complex in the LPS/TLR4 signal pathway. In agreement with these results we find that MAST205 forms a complex with TRAF6, regulating both the expression of MAST205 and the activity of TRAF6. Furthermore, FcγR ligation results in the rapid disappearance of MAST205 due to targeted degradation by the proteasome, a mechanism used to control expression of many short-lived proteins that control the cell cycle, transcription, and inflammation (9, 10). We report that MAST205 degradation is determined by the N-terminal domain and is regulated by phosphorylation. These results provide a clear mechanistic link between the adaptive and innate immune response.
from Sigma. A rabbit anti-MAST205 serum was generated by immunization with a 45-kDa glutathione S-transferase fusion protein containing amino acids 241–340 of MAST205. Antibodies against β-actin and ubiquitin mAb P4D1 were obtained from Santa Cruz; the hemagglutinin (HA) mAb 12CA5 was from Roche Applied Science; the FLAG mAb M2 was from Sigma; the Myc antibody 9E10 is from Upstate Biotechnology. Antennapedia peptides were synthesized with the N-terminal antennapedia sequence RQIKIWFQNRRMKWKK followed by \textsuperscript{18SGT-ENVPDEEGRSPR} (peptide 1) or the \textsuperscript{18VSSCSSQEKHLHQ} (peptide 2). Peptides were synthesized on a Symphony (Protein Technologies) peptide synthesizer at a 25\% coupling efficiency, and purified by high pressure liquid chromatography on a C18 column developed with a 1–75% acetonitrile gradient. Purified peptides were lyophilized and stored at −20 °C under nitrogen. When required, peptides were dissolved in water to a final concentration of 1–3 mM. The identity of the peptides was verified by mass spectrometry.

**Plasmids**—MAST205 full-length cDNA or and truncated cDNA fragments were inserted into the mammalian expression vector pcDNA3.1. The HA-ubiquitin plasmid and HA-ubiquitin mutant plasmids K48R, K29R, and K63R were kindly provided by Dr. Ze’ev Ronai (Mount Sinai School of Medicine). Plasmids encoding TRAF2, TRAF6, and MyD88 K29R, and K63R were kindly provided by Dr. Ze’ev Ronai (Mount Sinai School of Medicine). Plasmids encoding TRAF2, TRAF6, and MyD88 were kindly provided by Dr. Ruslan Medzhitov (Yale School of Medicine).

Cell Lines—The RAW264.7 murine macrophage cell line (American Type Culture Collection) and 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. 293T cells were transiently transfected using DNA-calcium phosphate precipitate. For each transfection, 10–20 μg of plasmid was used.

**NF-κB Reporter Assay**—RAW264.7 cells were transiently transfected using Superfect (Qiagen). For each transfection, 2.5 μg of plasmid was mixed with 100 μl of Dulbecco’s modified Eagle’s medium (without serum and antibiotics) and 10 μl of Superfect reagent, incubated at room temperature for 10 min, mixed with 600 μl of Dulbecco’s modified Eagle’s medium complete medium, and immediately added to the cells in 6-well plates. Luciferase activity was measured 16–24 h later. When indicated, LPS (100 ng/ml) was added to the culture 26–12 h before harvest. The cells were extracted with reporter lysis buffer (Promega), and 20 μl of extract was assayed for luciferase as described (11). Cells were co-transfected with a constitutively active cAMP-dependent protein kinase A (PKA)-galactosidase reporter plasmid to normalize experiments for transfection efficiency. Western Blotting—Cell lysates and prestained molecular weight markers were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in TBST (Triton-containing Tris-buffered saline), incubated with various antibodies (1:2000) for 1–2 h, washed with TBST, and stained with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (1:5000). Immunoblots were subjected to SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with 10% nonfat milk in PBS, 0.5% Triton-100, and incubated overnight with rabbit anti-MAST205 serum, anti-Myc, or anti-HA mAb. After washing, blots were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody, rinsed extensively in phosphate-buffered saline containing 0.5% Triton-100 and developed with super-signal chemiluminescent substrate (Pierce).

**FcγR Ligation**—J774 cells were seeded on glass dishes coated with trinitrophenyl-bovine serum albumin (12), and 5 μg/ml anti-DNP IgG2b mAb U12.5 was added to cross-link FcγR.

**RESULTS**

**MAST205 Interacts with TRAF6**—Previous results suggested that the locus of MAST205 in the TRL4-signaling pathway was proximal to the IKK complex. A dominant negative (DN) N-terminal deletion mutant of MAST205, BX-MAST205, had no effect on NF-κB activation after ectopic expression of IKK-β (4), but BX-MAST205 inhibited NF-κB activation stimulated by LPS or a constitutively active TRIF4 chimera. To characterize the pathway in more detail, we co-transfected MAST205 with molecules known to play a role in the TLR4 signaling. Remarkably, we found that immunoprecipitation of Myc-MAST205 resulted in co-precipitation of AU1-TRAF6 and TRAF2 (Fig. 1A). In addition, co-expression of TRAF6 or TRAF2 with MAST205 resulted in a significant increase in MAST205 expression (Fig. 1B). Transfection of TRAF2 also resulted in an increase in MAST205 expression, although more modest than that seen with TRAF6. Transfection of TRAF6 or TRAF2 with MAST205 resulted with molecules known to play a role in the TLR4 signaling. Remarkably, we found that immunoprecipitation of Myc-MAST205 resulted in co-precipitation of AU1-TRAF6 and TRAF2 (Fig. 1A). In addition, co-expression of TRAF6 or TRAF2 with MAST205 resulted in a significant increase in MAST205 expression (Fig. 1B). Quantification of a series of co-transfection experiments of 293T cells with TRAF6 showed a 10–20-fold increase in expression of MAST205. Co-transfection of TRAF2 also resulted in an increase in MAST205 expression, although more modest than that seen with TRAF6. These results demonstrate that endogenously expressed TRAF6 and MAST205 interact.

**TRAF6-induced NF-κB Activity Is Inhibited by MAST205**—TRAF6 is an E3 ubiquitin ligase with a typical ring domain, and the enzyme functions together with an E2 complex consisting of Ubc13 and Uev1A to catalyze synthesis of Lys-63 linked polyubiquitin chains (8). The substrates for TRAF6 include TRAF6 itself (13) and the TAK1-associated proteins TAB2 and TAB3 (14). We next analyzed the effect of the MAST205-TRAF6 complex on TRAF6 activation of NF-κB. As expected, overexpression of TRAF6 resulted in a substantial (22-fold) stimulation of NF-κB reporter activity, which is independent of LPS stimulation. However, the TRAF6 activation of the NF-κB reporter was largely inhibited by co-expression of MAST205...
(88%) and the N-terminal BX-MAST205 deletion mutant (92%) (Fig. 2A). This result shows that the formation of a complex with MAST205 negatively regulates TRAF6 activity and also suggests that there is also a TRAF6 binding site in BX-MAST205 mutant.

Because these results point to the locus of MAST205 acting at the level of TRAF6, we would predict that NF-κB activation due to overexpression of upstream components in the signal pathway would also be inhibited by BX-MAST205. NF-κB activation in RAW264.7 cells observed after transfection of MyD88 and TIRAP was inhibited by co-transfection of BX-MAST205 as we would predict (Fig. 2B). In addition, NF-κB activation due to poly(I:C) stimulation, which activates TLR3, is inhibited by BX-MAST205 (Fig. 2C). Thus, MAST205 also plays a role in TLR3 signal pathways, which differ significantly from other TLRs in utilizing the kinase receptor interacting protein and the adapter TRIF (15, 16).

MAST205 Peptides Modulate NF-κB Activation and Alter Complex Formation with TRAF6—Protein transduction domains (17) have been used to deliver peptides and small proteins to cells by an energy- and receptor-independent mechanism with the aim of interfering in signaling processes. Examples include protein transduction domain peptides directed against STAT3 (18), extracellular signal-regulated kinase (19), and NF-κB (20). To analyze the importance of the N terminus of MAST205, we synthesized protein transduction domain MAST205 peptides targeting a high scoring PEST sequence, thought to control proteasomal degradation (21) (residues 218–233; peptide 1) and a potential TRAF2 binding epitope (residues 188–201; peptide 2). We analyzed the effect of these peptides on LPS-stimulated NF-κB reporter activation and found that peptide 1 inhibited LPS-induced NF-κB activity by 50%, whereas peptide 2 resulted in 20% stimulation (Fig. 3A). These results are comparable to those obtained previously using RNAi to target MAST205 in J774 cells and show that MAST205 is an appropriate target for modulation of inflammatory response by cell-permeable peptides.

As another test for complex formation between MAST205 and TRAF6, we analyzed the complex of MAST205 and TRAF6 by Superose 6 gel filtration chromatography. TRAF6 forms a trimer in solution (22), and hence, the predicted size of a TRAF6-MAST205 complex, assuming each TRAF6 monomer binds one molecule of MAST205, would be 789 kDa. After fractionation of lysates from 293T cells co-transfected with TRAF6 and MAST205, the two proteins were always found associated, eluting over a wide range of $M_r$ (Fig. 3B) with a
substantial amount in advance of the 699-kDa thyroglobulin marker. At the predicted Mr for the TRAF6 trimer no staining of TRAF6 was observed. We next analyzed the effect of peptide 1 on complex formation. Preincubation with peptide 1 (30 μM) resulted in a dramatic shift in the MAST205/TRAf6 complex to a higher Mr (Fig. 3C). This result demonstrates that the peptide alters the composition of the complex.

We next tested the effect of co-expression of MAST205 and peptide 1 on TRAF6 ubiquitination, since ubiquitination of TRAF6 is central to NF-κB activation. 293T cells were co-transfected with MAST205 and TRAF6 and analyzed for ubiquitination of TRAF6 after immunoprecipitation. Transfected TRAF6 was ubiquitinated (Fig. 3D), and after co-transfection with MAST205 the extent of ubiquitination was clearly increased. The result is consistent with the hypothesis that MAST205, acting as a scaffolding protein, facilitates trans-ubiquitination. However, the addition of peptide 1 inhibited TRAF6 ubiquitination. The localization of peptide 1 within the PEST sequence present in the N terminus of MAST205 is consistent with inhibition of ubiquitination, assuming that the N terminus is required for binding of E2/E3 enzymes required for TRAF6 ubiquitination.

In response to activation of TLRs or IL-1R, TRAF6 is ubiquitinated by K63 polyubiquitin chains, resulting in downstream activation of NF-κB (13, 23). We hypothesized that BX-MAST205 inhibition of NF-κB might be due to blockade of TRAF6 ubiquitination. To test this possibility we transfected 293T cells with TRAF6, BX-MAST205, and HA-ubiquitin and analyzed TRAF6 ubiquitination after immunoprecipitation of TRAF6 by blotting for HA. The BX-MAST205 deletion mutant inhibits ubiquitination of TRAF6 under conditions where there is no alteration in the levels of general HA-ubiquitin incorporated into proteins or in the expression of TRAF6 (Fig. 3E). We conclude that the MAST205 N-terminal domain is required to assemble complexes required for TRAF6 ubiquitination.

**Stabilization of MAST205 Protein by TRAF6**—We reported...
that FcR ligation resulted in rapid ubiquitination and proteasomal degradation of MAST205. In a series of transfection and transduction studies of MAST205 in both macrophages and 293T cells, we always observed a low level of MAST205 expression relative to other constructs driven by the same CMV or retroviral promoters. We transduced J774 cells with Myc-MAST205 incubated with cycloheximide to block de novo protein synthesis and determined the $t_{1/2}$ of Myc-MAST205 degradation (Fig. 4A). Even in the absence of FcR ligation, the rate of degradation of the protein was surprisingly rapid ($t_{1/2} = 35$ min) (Fig. 4A), and degradation was blocked by incubation with MG132, a proteasome inhibitor (Fig. 4A). Lactacystin, another proteasome inhibitor with greater specificity (24), also blocked degradation, but E-64d, TPCK, 4-aminidophenylmethanesulfonyl fluoride, O-phenanthroline, and caspase inhibitor II were without effect (not shown). Interestingly, co-transduction with TRAF6 in J774 cells stabilized MAST205 protein ($t_{1/2} = 77$ min) relative to the control (Fig 4, C and D). The actual $t_{1/2}$ of MAST205 in the presence of TRAF6 may be even longer. The retroviral transduction efficiency (assessed by a parallel green fluorescent protein transduction) is about 70%, and therefore, those cells transduced with MAST205, but not with TRAF6, in which the rate of degradation is more rapid, will contribute significantly to the overall rate of degradation.

Because we had found that knocking down MAST205 expression by RNAi targeting resulted in an inhibition of LPS-stimulated NF-kB reporter activity (4), we thought further study of the mechanism of MAST205 degradation was warranted. We have observed that after retroviral transduction of J774 cells, the BX-MAST205 deletion mutant, which lacks the N-terminal 416 amino acids, was expressed at far higher levels than is MAST205 or a mutant in which the ATP binding domain of the kinase was inactivated (Fig. 5A). Furthermore, the addition of MG132 resulted in a significant increase in the expression of the latter two proteins accompanied by a shift to higher $M_r$, consistent with ubiquitination. In contrast, the level of expression of BX-MAST205 was not altered by MG132 nor was any shift in $M_r$ seen. These results suggest that the reason for the dramatic difference in expression of the BX-MAST205 mutant compared with MAST205 was due to the lack of ubiquitination of BX-MAST205, which consequently was much more stable.

To confirm the hypothesis that BX-MAST205 is not ubiquitinated, we co-transfected 293T cells with Myc-labeled MAST205 or BX-MAST205 and HA-tagged ubiquitin, immunoprecipitated cell lysates with anti-Myc mAb, and immunoblotted the resulting precipitates with anti-HA mAb. MAST205 shows a low level of constitutive ubiquitination, which was greatly increased after a 2-h incubation with MG132 (Fig. 5B). Under the same conditions BX-MAST205 was not altered by MG132 nor was any shift in $M_r$ seen. Thus, we hypothesize that the N terminus of MAST205 controls the degradation of the kinase. However, ubiquitin can serve other functions besides triggering degradation, and not all proteins that are degraded by the proteasome are ubiquitinated. Polyubiquitination leading to protein degradation occurs predominantly through isopeptide linkage to ubiquitin lysine 48 and, secondarily, to lysine 29 (25). To prove conclusively that the ubiquitination of MAST205 leads to degradation, we examined the effect of a set of HA-tagged ubiquitin mutants in which the lysines 29, 48, and 63 were mutated to arginine. By immunoblotting lysates for HA reactivity (not shown) we confirmed that each of the HA-ubiquitin mutants was comparably expressed. Co-transfection of 293T cells with Myc-MAST205 and
SB203580, which inhibits Akt with an IC50 of 3 nun, an inhibitor of phosphatidylinositol 3-kinase, and of protein kinase B/Akt (26), we also tested the effects of wortman-
was without effect (Fig. 5
MAST205 relative to the control; the K63R ubiquitin mutant
K48R ubiquitin mutants increased the level of expression of the HA-ubiquitin mutants showed that both the K29R and K48R ubiquitin mutants increased the level of expression of MAST205 relative to the control; the K63R ubiquitin mutant was without effect (Fig. 5C).
Phosphorylation Controls the Rate of Degradation of MAST205—Ubiquitination, regulated by phosphorylation of Ser/Thr residues to create docking sites for E3 ubiquitin ligases, often serves to control degradation. Because FcγR ligation induces Ser/Thr kinase activity, we analyzed the role of phasatases in MAST205 degradation. We first tested the inhibitor of protein phosphatases 1 and 2a, okadaic acid. We reasoned that inhibition of dephosphorylation would result in more rapid MAST205 degradation if constitutive phosphorylation of Ser/Thr residues targeted degradation. Indeed, we observed that incubation with okadaic acid resulted in a dose-dependent decrease of MAST205 expression in macrophages transduced with MAST205, without any effect on actin expression (Fig. 6A).
The enhanced rate of degradation of MAST205 found after incubation with okadaic acid suggests that Ser/Thr kinase activity regulates degradation. Dibutyryl cyclic AMP, which activates protein kinase A, had no effect on degradation. However, incubation with phorbol myristate acetate significantly inhibited expression of transduced MAST205 in J774 cells. Ionomycin alone had a modest effect, and the combination of ionomycin and phorbol myristate acetate (PMA) resulted in slightly more inhibition than either alone (Fig. 6B). These results suggest strongly that an isomer of protein kinase C is responsible for the proteasome-mediated degradation of MAST205. Because FcγR ligation is reported to activate protein kinase B/Akt (26), we also tested the effects of wortman-
in, an inhibitor of phosphatidylinositol 3-kinase, and of SB203580, which inhibits Akt with an IC50 of 3–5 μM in addition to inhibiting p38 mitogen-activated protein kinase at a 10-fold lower concentration (27). Neither inhibitor had any effect on the level of expression of MAST205 with or without MG132 (Fig. 6C).
The N-terminal Domain of MAST205 Controls Degradation—The lack of ubiquitination of the BX-MAST205 deletion mutant suggests that sequences controlling degradation reside within the N-terminal 416 amino acids. Because sequences rich in proline, glutamic acid, serine, and threonine (PEST sequences) often target proteins for degradation (21, 28), we analyzed this domain of MAST205 using a web-based algo-
rithm, PESTfind. Residues 216–228 constitute a potential PEST domain, with a highly significant score of 5.45. Furthermore, Ser/Thr residues in and adjacent to PEST domains can function as sites for phosphorylation and create docking sites for E3 ubiquitin ligases. The dramatic increase in expression of

**Fig. 5.** MAST205 is ubiquitinated and degraded by the proteasome; the BX-MAST205 deletion mutant is not. A, J774 cells were transduced by retrovirus encoding the proteins as indicated for 48 h and then incubated with and without MG132 (20 μM) for 2 h. Aliquots of cell lysates (20 μg of protein) were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted for Myc, GFP, green fluorescent protein. B, 293T cells were co-transfected with pcDNA3-based vectors encoding Myc-MAST205 or Myc-BX-MAST205 and HA-ubiquitin; 48 h later cell lysates were prepared and immunoprecipitated with anti-Myc mAb 9E10. The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for HA with mAb 12CA5. C, 293T cells were cotransfected with vectors encoding Myc-MAST205 and HA-tagged ubiquitin mutants K29R, K48R, and K63R for 48 h. Cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted for Myc.

the BX-MAST205 deletion mutant and the lack of ubiquitina-
tion of this protein suggested that the N terminus, deleted in BX-MAST205, is responsible for the rapid degradation of the full-length protein. To confirm this hypothesis, we constructed a set of retroviral vectors encoding Myc-tagged N-terminal fragments (Fig. 7A). When transduced into J774 cells, these constructs expressed proteins of appropriate size, and both the expression and Mw of the fragments were increased after a 2-h incubation with MG132 (Fig. 7B). These results are similar to those shown in Fig. 5A, demonstrating the effect of incubation with MG132 on MAST205. MAST205 degradation is accelerated by FcγR ligation. We then confirmed that the N-terminal fragments behaved similarly. We transduced J774 cells with
A MAST205 Complex with TRAF6 Regulates NF-κB Activation

these constructs, full-length MAST205, and the BX-MAST205 mutant and plated the cells onto glass plates coated with trinitrophenyl-bovine serum albumin. The FcγRs were engaged by the addition the IgG2b anti-DNP mAb U12.1. BX-MAST205 expression was not altered by FcγR ligation, but expression of MAST205, the Δ418, and the Δ307 MAST205 N-terminal constructs was inhibited by FcγR ligation (Fig. 7C).

Given the similarity in ubiquitination and degradation of the N-terminal fragments and full-length MAST205, we next examined the effect of TRAF6 on the expression of the N-terminal fragments of MAST205. To some extent, co-expression of TRAF6 increased the level of expression of all the N-terminal fragments but had the most dramatic effect on Δ335, which was expressed very poorly relative to the other fragments (a longer exposure of Δ335 is shown) (Fig. 7D). These results imply that a site in the N terminus interacts with TRAF6. The dramatic increase in expression of BX-MAST205 relative to MAST205 (Figs. 5A and 7C) and the localization of the site for ubiquitination in the N terminus highlight the importance of this domain for MAST205 function.

**DISCUSSION**

We have recently identified MAST205 as a regulatory protein in the TLR2/4 pathways leading to activation of NF-κB and synthesis of IL-12. Inhibition of MAST205 function either by expression of DN-MAST205 constructs or by RNAi targeting of MAST205 results in the inhibition of IL-12 synthesis and of NF-κB reporter activity (4). FcγR ligation triggers MAST205 proteasomal degradation, which we suggest may explain the inhibition of macrophage LPS-stimulated IL-12 synthesis after incubation with immune complexes observed by Mosser and co-workers (7, 29–34) and confirmed by us. However, the exact position of MAST205 in the signal transduction pathway was not clear. Epistasis experiments suggested that MAST205 interacted with components upstream of the IKK complex because DN-MAST205 had no effect on NF-κB activation resulting from overexpression of IKK-β, the predominant IKK kinase activated by LPS (35). We now report that MAST205 forms a complex with TRAF6, and, consequently, TRAF6 activity is inhibited, and the stability of MAST205 is increased. Activation of NF-κB by poly(I:C) or by overexpression of adapter molecules TIRAP and MyD88 is inhibited by BX-MAST205. These results further support our previous results that MAST205 is an important component in TLR signaling, acting proximal to the IKK complex.

The striking inhibition of NF-κB activity we observe when TRAF6 is overexpressed with MAST205 or BX-MAST205 suggests that the basic function of MAST205 is to regulate TRAF6, controlling the activity of this potentially dangerous enzyme. If this theory is correct, how can it be explained that the data showing that RNAi targeting MAST205 inhibits LPS activation of NF-κB (4)? We hypothesize that MAST205, TRAF6, and other associated proteins form a complex to transmit signals from the TLR through the interleukin-1 receptor-associated kinase complex to the IKK complex. We suggest that the release of the inhibition of TRAF6 after binding of LPS is due to MAST205 kinase activity, phosphorylating downstream components, perhaps including TRAF6. Supporting this thesis, the ATP binding domain mutant of MAST205, inactive as a kinase, acts as a dominant negative for LPS-induced NF-κB activation (4). In this model, MAST205 kinase activity would be regulated by signals from upstream components in the pathway. Indeed, there is precedent for modulation of MAST205 kinase activity, which is regulated during sperm maturation (36). Characterization of the proteins involved in the TRAF6-MAST205 complex and identification of the substrates of MAST205 is a high priority.

TRAF6 is an ubiquitin ligase that is pivotal in the signal transduction pathways leading from Toll receptors, IL-1 receptor, and CD40. The presence of a MAST205-TRAF6 complex was demonstrated both by immunoprecipitation and by gel filtration. TRAF6 activation is reported to be controlled by two complexes, TRIKA1, composed of Ubc13 and Uev1A, and
TRIKA2, composed of TAK1 and adaptor proteins TAB1 and TAB2, which together with TRAF6 catalyze the synthesis of Lys-63-linked polyubiquitin chains and activate TAK1 (13). A second complex between TAK1, TAB1, and TAB3 was recently reported (37, 38) along with the observation that agonist (IL-1, tumor necrosis factor-α, LPS) activation of TAK1 was associated with Ser/Thr phosphorylation. TAB2 and TAB3 are ubiquitinated by TRAF6 in response to IL-1 and tumor necrosis factor-α (14). TRAF6 itself is activated by ubiquitination (23). We find, remarkably, that the BX-MAST205 deletion mutant inhibits TRAF6 ubiquitination, which provides a mechanistic explanation for the inhibition of this mutant on NF-κB activation by LPS.

Comparison of MAST205 with orthologues from Caenorhabditis elegans and Drosophila reveals three well conserved domains: the N terminus, the protein kinase A/C domain, and a PDZ domain. We believe MAST205 acts as a scaffolding protein that coordinates complexes by binding to the PDZ and the PDZ domain. We believe MAST205 acts as a scaffolding protein mains: the N terminus, the protein kinase A/C domain, and a ditis elegans...

Hence, small molecule inhibitors of the MAST205 regulation of T cell, B cell, and dendritic cell responses, since in these cells a role for TRAF6 has been definitively shown (45–47). Our future study will focus on how MAST205 affects the adaptive immune response by controlling the differentiation of naïve T cells into T helper 1 T cells and the maturation of dendritic cells. We believe that MAST205, a large protein with multiple protein/protein interaction domains and a kinase activity that is required for its function, offers an attractive target for therapeutic intervention at multiple levels.

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