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Serpin 2a Is Induced in Activated Macrophages and Conjugates to a Ubiquitin Homolog[1]

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After i.p. infection of mice with the intracellular bacterium *Mycobacterium bovis* bacillus Calmette-Guérin, macrophages recovered from the peritoneal cavity display classical signs of immune activation. We have identified a member of the serine protease inhibitor (serpin) family which is highly induced in macrophages during bacillus Calmette-Guérin infection. Serpin 2a (spi2a) expression is also induced in macrophages in vivo during infection with *Salmonella typhimurium* and *Listeria monocytogenes*, and in vitro by a variety of bacteria and bacterial products. The cytokine IFN-γ also induces spi2a expression in macrophages, and this induction is synergistic with bacterial products. We also demonstrate here that a ubiquitin homolog, IFN-stimulated gene of 15-kDa (ISG15), is strongly induced during in vitro and in vivo activation of macrophages and that it conjugates to spi2a in activated macrophages. The ISG15-spi2a conjugates were identified by tandem mass spectrometry and contained spi2a conjugated to either one or two molecules of ISG15. Whereas spi2a was induced by either bacterial products or IFN-γ, ISG15 was induced only by bacterial products. Although many protein targets have been described for ubiquitin conjugation, spi2a is the first ISG15-modified protein to be reported. Macrophage activation is accompanied by the activation of a variety of proteases. It is of interest that a member of the serine protease inhibitor family is concomitantly induced and modified by a ubiquitin-like protein. ***The Journal of Immunology*, 2002, 168: 2415–2423.**

Macrophages play a critical role in host defense against bacterial pathogens. Their bactericidal activity is induced by bacterial products and is a result of a complex interplay between elements of the innate and adaptive immune system (1, 2). At least three criteria must be met for the macrophage to efficiently kill the bacterium and activate the adaptive immune system by a process known as Ag presentation. Macrophages must increase the production of bactericidal agents including reactive nitrogen and reactive oxygen intermediates, upregulate a variety of proteases that can degrade the dead bacteria, and express higher levels of surface MHC class II molecules that can present the resultant bacterial peptides to T cells (reviewed in Refs. 1 and 3). Although activated macrophages are very efficient at killing bacteria, the task of Ag presentation falls largely on the related dendritic cells (4).

The activation of macrophages and dendritic cells in vivo is complex (5). Using cDNA arrays, we investigated the changes in gene expression associated with macrophage activation during in vivo infection of mice with the intracellular bacterium *Mycobacterium bovis* bacillus Calmette-Guérin (BCG),[4] a classical system for studying immune macrophage activation. In this screen, we identified serpin 2a (spi2a) as a protein with substantially increased expression during BCG infection in vivo (6, 7). Serpins are a protein superfamily with conserved structure that regulate both serine and cysteine protease function in diverse processes including coagulation, extracellular matrix degradation, complement activation, fibrinolysis, and apoptosis (8, 9). We report here that spi2a is increased >100-fold not only during in vivo activation of macrophages by BCG but also during infection of mice with *Listeria monocytogenes* and *Salmonella typhimurium*. *In vitro*, bacteria and bacterial products, as well as the cytokine IFN-γ, induce the spi2a promoter, and the combination of these macrophage activators is synergistic for spi2a induction. spi2a is also induced in dendritic cells by bacterial products. Our data suggest that spi2a regulates intracellular proteases in activated APC.

While studying the expression of spi2a in macrophages by Western blotting, we detected spi2a not only at its predicted molecular mass but also in more slowly migrating forms. These species proved to be spi2a conjugated to a ubiquitin homolog known as IFN-stimulated gene of 15-kDa (ISG15) (10). ISG15, also known as ubiquitin cross-reactive protein, is induced by type I IFNs in a variety of cell types (11–13) and conjugates to intracellular proteins in a process analogous to that for ubiquitin (14). We report here the first identification of a substrate for ISG15 conjugation. ISG15 conjugation to spi2a occurs in macrophages that have been activated by incubation with bacterial products. Additionally, in vitro incubation with LPS as well as in vivo infection

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[4] Abbreviations used in this paper: BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; spi2a, serpin 2a; ISG15, IFN-stimulated gene of 15-kDa; His tag, histidine tag; HPRT, hypoxanthine phosphoribosyltransferase; AraLAM, lipoarabinomannan; mAGP, mycolylarabinogalactan-peptidoglycan complex; iNOS, inducible NO synthase; SCWP, soluble cell wall proteins.

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with BCG potently induces the expression of both spi2a and ISG15 protein.

Materials and Methods

Cells and mice

RAW264.7 macrophages (American Type Culture Collection (ATCC), Manassas VA) were cultured in RPMI (BioWhittaker, Gaithersburg, MD) with 10% FCS (HyClone Laboratories, Logan, UT), glutamine, penicillin, and streptomycin (Life Technologies, Gaithersburg, MD) at 37°C and 5% CO_2. Female ICR and C57BL/6 mice were purchased from Charles River Breeding Laboratories (Wilmington, MA).

Bacteria and infection

Mycobacterium bovis BCG (strain Pasteur; ATCC) was a gift from Dr. S. Smith (University of Washington, Seattle, WA). BCG was grown in Proskauer-Beck medium with aeration to 5×10^7 CFU/ml and stored in aliquots at −70°C. To infect mice, an aliquot of BCG was thawed, sonicated three times for 30 s in a water bath sonicator, and diluted in PBS. Mice were injected i.p. with 5×10^6 CFU. *Listeria monocytogenes*, strain 10403S, was grown in trypticase soy broth (Difco, Detroit, MI). *Listeria* in log phase were diluted in PBS, and 1×10^3 CFU were injected i.p. *S. typhimurium* SL3261 (attenuated DL1344 ΔaroAhisGsylrpsL) was a gift from Dr. B. Cookson (University of Washington). *Salmonella* growing at log phase in LB (Difco) were diluted in PBS, and 1×10^5 CFU/mouse were injected i.p.

Macrophages and dendritic cells

Activated macrophages were harvested by peritoneal lavage with PBS 12 days after infection with BCG and 5 days after infection with *Listeria* or *Salmonella*. Resident peritoneal macrophages were harvested from uninfected ICR mice. Unless otherwise noted, macrophages were plate adhered for 2 h and then washed several times with PBS to remove nonadherent cells. Cells remaining were >95% macrophages by visual inspection. Bone marrow dendritic cells were generated by a modification of the method of Inaba et al. (15). Briefly, bone marrow cells from C57BL/6 mice were cultured with 20 ng/ml rGM-CSF (R&D Systems, Minneapolis, MN) for 7 days. Dendritic cells were then cultured in medium or in medium with 100 ng/ml LPS (List Biological Laboratories, Campbell, CA) for 24 h; stained with Abs to CD11b, CD11c, and IA^b; and purified by sorting on a FACSVantage (BD Biosciences, San Jose, CA). Unstimulated dendritic cells were sorted as $CD11b^+CD11c^+IA^{b-}$ cells, whereas LPS-stimulated dendritic cells were sorted as $CD11b^+CD11c^+IA^{b+}$ cells, using class II MHC up-regulation as a marker of activation.

cDNA arrays

Macrophages from day 12 after BCG infection were lysed in Trizol (Life Technologies), and then total RNA was isolated according to manufacturer's instructions. mRNA was then purified using two rounds of oligo(dT) cellulose columns (Pharmacia, Piscataway, NJ); 5 μg of this mRNA were used as a template to generate an unamplified oligo(dT)-primed cDNA library in the pSPORT plasmid vector according to the manufacturer's directions (Life Technologies). Then 9200 individual clones in *E. coli* were grown and stored in 384-well plates. These clones were then spotted using a Q-bot onto 20- × 20-cm nylon membranes in duplicate, with the central spot of every grid of 9 containing a plasmid with a control cDNA. The colonies were lysed on the membrane using proteinase K, and the plasmid DNA was denatured, neutralized, and cross-linked to the membrane using a Stratalinker (Stratagene, La Jolla, CA). Duplicate membranes were probed with ^{32}P-labeled first strand cDNA generated from mRNA from resident peritoneal or BCG macrophages primed with a mixture of oligo(dT) and random hexamer primers. Control mRNA was added to the labeling reactions to control for labeling and hybridization efficiency between probes and membranes, respectively. Hybridization was detected using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager, and the location and intensity of individual spots were determined using SFV software provided by Dr. R. Baumgartner. Average background for the membrane was subtracted from the spot intensities, and intensities were normalized for average control spot intensity. Duplicate spots on each membrane for each clone were averaged, and data were discarded if the duplicate intensities varied by >50%. Clones that met this criterion and that showed a >2-fold difference in intensity were sequenced. Sequences were analyzed by searching the nonredundant and EST databases at GenBank using the Advanced Blast search algorithm.

Northern blot

Total RNA from resident peritoneal and BCG-activated macrophages was isolated as above. For in vitro activated macrophages, resident peritoneal macrophages were plate adhered overnight, nonadherent cells were removed, and cells were activated with 100 ng/ml LPS or 10 U/ml IFN-γ (R&D Systems). After 4 h (LPS) or 48 h (IFN-γ), cells were lysed, and RNA was prepared as above. Northern blot analysis of 10 μg total RNA was conducted using standard methods. The Northern blot was probed with a random primed, [^{32}P]dCTP (New England Nuclear, Boston, MA)-labeled probe corresponding to the entire cDNA for spi2a or for *EF1α* as a housekeeping gene.

Expression constructs and transfection

All expression constructs were in the EF6/V5-His-TOPO vector (Invitrogen, San Diego, CA). The spi2a-HA construct was generated by amplifying the spi2a open reading frame using a forward primer encoding an HA tag N-terminal to the cDNA start (forward primer, CGGAATTCATGTAC CCATACGACGTCCCAGACTACGCTGCTGGTGTCTCCCCTGCTGTC; reverse primer, CGGGATCCTCACTGTCCAATCAGGCATAG) with the cDNA array clone as template. The spi2a-HisHA expression construct was generated with the identical reverse primer to the spi2a-HA construct and with a forward primer encoding both the 6-residue histidine tag (His tag) and the HA tag (forward primer, ATGCATCATCACCATCACCATTACCCATAC GACGTCCCAGACTACGCTGCTGGTGTCTCCCCTGCTGTC). To generate the ISG15-V5 construct, the open reading frame of ISG15 was amplified from cDNA made from total RNA of RAW264.7 cells treated for 4 h with 100 ng/ml LPS (List) using a forward primer encoding a V5 tag N-terminal to the start codon (forward primer, ATGGGTAAGCCTATC CCTAACCCTCTCCTCGGTCTCGATTCTACGGCCTGGGACCTAAA GGTG; reverse primer, TTAGGCACACTGGTCCCCTCC). All constructs were verified by sequence analysis. Ten micrograms DNA were transiently transfected into between 10 and 50×10^6 RAW264.7 cells by electroporation (16). Cells were plate adhered overnight and used for subsequent experiments. To generate stable clones, transient transfectants were selected in medium with 5 μg/ml blasticidin (Invitrogen) for 10 days and then cloned by limiting dilution.

Western blot

Approximately 5×10^5 transiently transfected cells or stable clones were plated in wells of 24-well dishes. In some cases, cells were treated overnight with 100 ng/ml *Salmonella minnesota* LPS (List). The cells were lysed in 10 mM HEPES, pH 7.4, with 150 mM NaCl and 1% Triton X-100 containing leupeptin (1 μM, Boehringer Mannheim, Indianapolis, IN), aprotinin (1/100, Sigma, St. Louis, MO), and PMSF (1 mM, Boehringer Mannheim). The extracts were spun at 15,000 rpm for 15 min to remove nuclei, and then the supernatant removed for analysis by SDS-PAGE. Resident peritoneal macrophages were plated at $\sim 1 \times 10^6$ macrophages per well in 24-well tissue culture dishes. After overnight adherence, nonadherent cells were washed away, and then cells were cultured with medium alone or medium with LPS at 100 ng/ml or IFN-γ at 10 U/ml (R&D Systems). At the indicated times after activation, the cells were lysed, and cytoplasmic extracts generated as above. For the experiment involving in vivo activated macrophages, macrophages from uninfected mice or those from BCG-infected mice were plate adhered for 2 h before removal of nonadherent cells and lysis. Extracts were prepared as above. A 25-μg protein sample was used per lane for this experiment. For detection of the tagged proteins, the anti-HA.11 mAb (Covance, Princeton, NJ) or the anti-PK mAb to the V5 tag (Serotech, Raleigh, NC) was used as suggested by manufacturer with anti-mouse HRP (Zymed Laboratories, San Francisco, CA) and detected using ECL Plus (Amersham, Arlington, Heights, IL). To detect ISG15, Western blots were probed with affinity purified rabbit polyclonal antiserum to ISG15 (14) used at 1 μg/ml and anti-rabbit HRP (Zymed Laboratories). To detect spi2a, Western blots were probed with affinity-purified rabbit polyclonal antiserum generated to recombinant spi2a (E. C. Morris, T. Dafforn, S. L. Forsyth, A. J. Horvath, L. Hampson, I. N. Hampson, R. W. Carrell and P. B. Coughlin, manuscript in preparation) used at 1/1000 dilution followed by anti-rabbit HRP.

Semiquantitative RT-PCR

Approximately 1 μg total RNA from macrophage populations was reverse transcribed using Superscript II reverse transcriptase and oligo(dT) primers (Life Technologies). Serial 1/3 dilutions of cDNA were amplified with primers to murine hypoxanthine phosphoribosyltransferase (HPRT) to standardize between cDNA samples (forward primer, GATACAGGC CAGACTTTGTTG; reverse primer, GGTAGGCTGGCCTATAGGCT). Matched 3-fold dilutions of cDNA from each sample were then amplified

with primers to spi2a (forward primer, GGAATGGCAGGTGTCGGATG; reverse primer, GGTCAGGAACCTGATTTCGTC). These primers were chosen to minimize cross-hybridization with other serpins that may be expressed in macrophages; the forward primer encompasses the reactive site loop of spi2a, and the reverse primer is in the 3′-UTR. Amplified products were separated on 1% agarose gels and visualized with ethidium bromide staining.

Real time PCR

cDNA from dendritic cells purified by cell sorting was generated as above and amplified with probe and primer sets for murine HPRT and spi2a as indicated below using TaqMan Universal PCR master mix and an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Genomic DNA contamination was measured by including template that had been mock reverse transcribed and at all times accounted for <10% of the signal. Fold induction was determined from C_t values normalized for HPRT expression and then normalized to the value derived from medium-treated dendritic cells. Primers and probes used were as follows. Spi2a: forward, CCAAATGGTGAGG GTGCTTCT; reverse, GCATAGCGGATCACCAAAACA; probe, CCCA ACGGCTGGAATCTAAGCGTTTAT. HPRT primers: forward, TGGAA AGAATGTCTTGATTGTTGAA; reverse, AGCTTGCAACCTTAACCA TTTTG; probe, CAAACTTTGCTTTCCCTGGTTAAGCAGTACAGC.

Luciferase assays

RAW 264.7 cells (1×10^7) were transiently transfected by electroporation as above with 10 μg pXP-2 plasmid (17) containing −259 to +46 nucleotides of the spi2a promoter driving the firefly luciferase gene (18) and plated into one 96-well dish. Cells were adhered overnight, washed once with PBS, and treated as indicated with IFN-γ at 10 U/ml (R&D Systems) or medium for 8 h. Bacteria or their products were then added for 14 h at the indicated final concentration. All stimuli except LPS and *S. minnesota* were treated with polymyxin B (10 μg/ml) for 1 h before addition to cells to ensure that the data did not result from LPS contamination. Cells were lysed, and luciferase activity was read using the Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Heat-killed *Mycobacterium tuberculosis* H37Rv (100 μg/ml) and *M. tuberculosis* products lipoarabinomannan (AraLAM; 10 μg/ml), mycolylarabinogalactan-peptidoglycan complex (mAGP; 100 μg/ml), and soluble cell wall proteins (SCWP; 1 μg/ml) were obtained from J. Belisle (National Institute of Allergy and Infectious Diseases, Bethesda, MD) through the TB Research Materials and Vaccine Testing Contract. Heat-killed *Staphylococcus aureus* (clinical isolate) was used at 2×10^6 bacteria/well, and heat-killed *S. minnesota* R595 (ATCC) was used at 2×10^3 bacteria per well. Zymosan (3×10^6 particles/well) was obtained from Molecular Probes (Eugene, OR), *S. minnesota* LPS (100 ng/ml) was obtained from List, *Staphylococcus aureus* peptidoglycan (10 μg/ml) was from Fluka (Buchs, Switzerland), and the synthetic lipopeptide PAM_3CSK_4 (100 ng/ml) was from Boehringer Mannheim.

Purification of spi2a-HisHA and identification of complexed proteins

For affinity purification, CNBr-activated Sepharose beads conjugated to the HA.11 mAb at 1 mg Ab/ml beads following the manufacturer's instructions (Pharmacia) were used followed by Ni^{2+} beads (Invitrogen). Cytoplasmic extracts generated in 20 mM phosphate buffer, pH 7.8, with 150 mM NaCl, 1% Triton X-100 (lysis buffer), and protease inhibitors as above were pooled from 2×10^9 RAW264.7 macrophages transiently transfected with the spi2a-HisHA construct. The extracts were incubated with HA.11 beads for 1 h at 4°C with rocking. The HA.11 beads were washed three times with lysis buffer without Triton X-100 and eluted by boiling in this buffer with 0.3% SDS to prevent precipitation of proteins. This eluate was then incubated with Ni^{2+} beads at room temperature for 1 h, washed five times in 20 mM phosphate buffer, pH 7.8, with 150 mM NaCl and then three times with 20 mM phosphate buffer, pH 6.0, with 150 mM NaCl. The protein was eluted by boiling in SDS sample buffer containing 2-ME. Ninety-five percent of the eluted protein was run in one lane of a 10% SDS-PAGE gel for silver stain, and the remaining 5% was run in one lane for Western blotting with the HA.11 Ab. Silver staining was performed as described (19).

To prepare samples for tandem mass spectrometry, silver-stained bands were excised from the gel, cut into ~1-mm cubes, and subjected to in-gel tryptic digests (19). Peptides generated were identified by nanoscale microcapillary liquid chromatography-tandem mass spectrometry techniques using an LCQ Classic ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) (20). Spectra were searched against the OWL nonredundant protein sequence database as well as the EST databases using the program SEQUEST (21), which matches theoretical and acquired tandem mass spectra.

Results

Spi2a is up-regulated in macrophages during bacterial infection in vivo

We used cDNA arrays to identify a large number of genes that are specifically induced in murine peritoneal macrophages during in vivo infection with BCG (data not shown). One of these genes encoded serpin 2a (spi2a), a member of the serine protease inhibitor (serpin) family. By Northern blot analysis, mRNA for spi2a was not detected in resident peritoneal macrophages from uninfected mice but was detected strongly in macrophages from mice infected for 12 days with BCG (Fig. 1*A*). The strong induction in BCG-activated macrophages was also seen at the protein level; Fig. 1*B* shows a Western blot of macrophage lysates probed with polyclonal antiserum recognizing spi2a. The specificity of the polyclonal antiserum to spi2a is also shown in Fig. 1*B*. The polyclonal serum detects HA-tagged spi2a when transfected into the

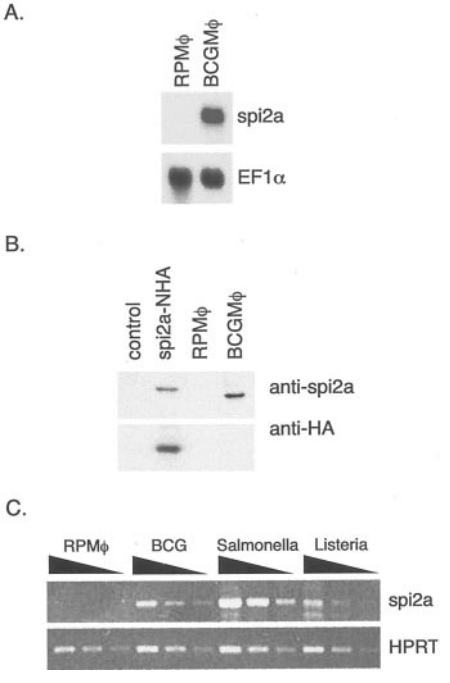

FIGURE 1. Spi2a is up-regulated in macrophages induced in vivo by bacterial infection. *A*, *Top*, Northern blot analysis of total RNA from resident peritoneal macrophages from uninfected mice (RPMϕ) and peritoneal macrophages from mice infected 12 days previously with BCG (BCGMϕ) probed with the complete cDNA for spi2a; *bottom*, the same blot stripped and reprobed for the housekeeping gene *EF1α*. *B*, *Top*, Western blot analysis of spi2a expression in cytoplasmic extracts from peritoneal macrophages as in *A* or from vector control (control) and HA-tagged spi2a (spi2a-NHA) expression construct-transfected RAW264.7 macrophages. spi2a protein is detected using polyclonal antiserum generated to recombinant spi2a protein. *Bottom*, The same blot stripped and reprobed with a mAb to the HA epitope tag. The slower mobility of the transfected spi2a in comparison with endogenous spi2a is due to the addition of the HA epitope tag. *C*, Semiquantitative RT-PCR analysis of cDNA generated from total RNA from resident peritoneal macrophages, macrophages isolated 5 days after *Salmonella* and *Listeria* infection, and 12 days after BCG infection. Three-fold serial dilutions of cDNA were matched for HPRT expression (*bottom*) and then amplified with primers for spi2a (*top*). PCR products were separated on agarose gels and detected by ethidium bromide staining.

macrophage cell line, RAW264.7, but not in vector control-transfected cells, which do not express any endogenous spi2a mRNA (Fig. 1*B* and data not shown). The band detected by the polyclonal serum is identical with that detected by an anti-HA Ab on the same blot after stripping (Fig. 1*B*).

We investigated whether induction of spi2a was unique to infection with *Mycobacterium* or whether infection with other intracellular pathogens would also induce this transcript. Mice were infected i.p. with *L. monocytogenes*, a Gram-positive bacterium, or an attenuated strain of *S. typhimurium*, a Gram-negative bacterium, and peritoneal macrophages were isolated after 5 days. spi2a mRNA levels were assessed by semiquantitative RT-PCR and compared with resident peritoneal macrophages and BCG macrophages. Fig. 1*C* shows that both infection with *S. typhimurium* and infection with *L. monocytogenes* induced spi2a mRNA. This suggests that induction of spi2a is a general response of macrophages to in vivo infection with intracellular bacteria and that it is not specific to mycobacterial infections.

spi2a is induced in vitro by bacterial products and IFN-γ

We assessed whether the induction of spi2a was in response to the bacteria directly or indirectly via a cytokine induced in vivo by bacterial infection. To address this, we initially performed Northern blot analysis on total RNA from resident peritoneal macrophages that were treated in vitro with LPS, a molecule from the coat of Gram-negative bacteria, or with IFN-γ, a cytokine that is secreted by NK cells and T cells in response to bacterial infection. These molecules are both potent activators of macrophages although they act on distinct pathways. Both LPS treatment for 4 h and IFN-γ treatment for 48 h induced spi2a mRNA in resident peritoneal macrophages to a greater extent than culture alone (Fig. 2*A*). We also investigated whether spi2a is similarly regulated in myeloid dendritic cells, a cell highly related to the macrophage. Real time PCR analysis showed that spi2a mRNA is also induced by LPS treatment in bone marrow-derived dendritic cells, a stimulation that also induces maturation of these cells and up-regulation of cell surface class II MHC levels (Fig. 2*B*). These data suggest that spi2a is regulated similarly in macrophages and dendritic cells.

The finding that LPS, a Gram-negative bacterial product, can activate transcription of spi2a in vitro led us to ask whether other classes of bacteria and their products can also induce spi2a. To do so, we used the RAW264.7 macrophage cell line transfected with a plasmid containing base pairs −259 to +46 of the spi2a promoter driving the firefly luciferase gene as a reporter (18). LPS treatment of RAW264.7 cells induces spi2a mRNA as is seen in resident peritoneal macrophages (data not shown). A variety of whole bacteria as well as purified bacterial cell wall components and yeast cell walls directly induced the spi2a promoter in vitro (Fig. 2*C*). This induction varied between 2.5-fold (araLAM) and 4.6-fold (zymosan) over basal levels. As found with in vivo infection, Gram-negative (*S. minnesota*) and Gram-positive (*Staphylococcus aureus*) bacteria as well as *Mycobacterium* (*M. tuberculosis*) activated this promoter. The bacterial products tested were derived from Gram-negative bacteria (LPS), Gram-positive bacteria (peptidoglycan) and *Mycobacterium* (araLAM, MAGP, SCWP). The doses used in this experiment have been previously shown to induce maximal production of TNF-α from RAW264.7 cells (data not shown), and all stimuli except LPS and *Salmonella* were treated with polymyxin B to ensure the induction did not result from LPS contamination.

IFN-γ is known to sensitize macrophages to respond to bacterial stimuli. Therefore, we were interested in how the presence of IFN-γ affected the ability of macrophages to induce spi2a in response to these stimuli. To test this, we pretreated RAW264.7 macrophages transfected with the spi2a promoter luciferase construct with IFN-γ and then activated with bacteria

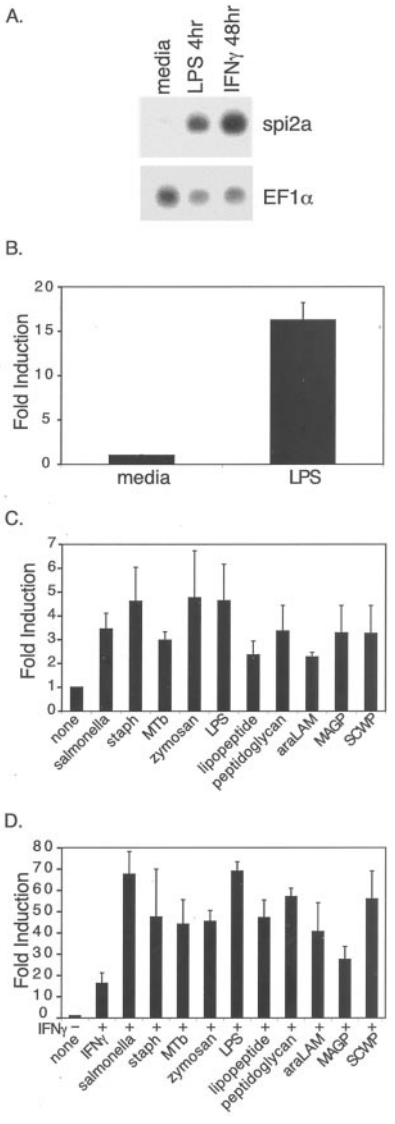

FIGURE 2. spi2a is induced in macrophages and dendritic cells by in vitro treatment with bacterial products and IFN-γ. *A*, Northern blot analysis of resident peritoneal macrophages that had been plate adhered (medium) or activated with LPS for 4 h (LPS 4 h) or IFN-γ for 48 h (IFN-γ 48 h). Spi2a and *EF1α* transcripts were detected as in Fig. 1*A*. *B*, Real time PCR analysis of spi2a expression in bone marrow-derived dendritic cells. Dendritic cells from a 7-day culture of GM-CSF-treated bone marrow were either cultured in medium or with 100 ng/ml LPS for 24 h and then sorted as $CD11b^{+}CD11c^{+}$ cells that were either IA^{b-} (medium) or IA^{b+} (LPS). cDNA from these populations was subjected to real time PCR using primer/probe sets specific for spi2a and HPRT and normalized to HPRT levels. Data are represented as the fold induction of spi2a over medium-treated cells and are the average of triplicate samples ± SD. *C*, RAW264.7 cells were transfected with a plasmid containing bases −259 to +46 of the spi2a promoter driving the firefly luciferase gene. The cells were stimulated with bacteria or their products as indicated for 14 h and lysed, and the luciferase activity was measured. The amount of each stimuli used is listed in *Materials and Methods*. The activity of the promoter is represented as the fold induction over untreated cells (none) on the *y*-axis. *D*, Cells were transfected and treated as in *B* but were first pretreated with IFN-γ (10 U/ml) for 8 h before the addition of stimuli. The data in *C* and *D* are expressed as the mean of three independent experiments ± SEM. staph, *Staphylococcus aureus*; MTb, *M. tuberculosis*.

or bacterial components as in Fig. 2*C* before assaying luciferase activity. As shown in Fig. 2*D*, IFN-γ alone induces the spi2a promoter in this system as it does in primary macrophages (Fig. 2*A*). The induction by IFN-γ is 16-fold in the RAW264.7 system, which is greater than the extent of induction by bacterial products alone seen in Fig. 2*C*. Interestingly, pretreatment with IFN-γ caused a large increase in the induction of the spi2a promoter by the bacteria and their components (Fig. 2*D*). This induction ranged from 27-fold (MAGP) to 68-fold (LPS) over background and was much greater than the additive effects of IFN-γ and the bacteria/components alone. These results were confirmed when looking at protein production by primary macrophages treated in vitro with LPS either with or without IFN-γ pretreatment for 14 h (data not shown). Therefore, as with other proteins important in the macrophage response to pathogens such as inducible NO synthase (iNOS) and IL-12, there is profound induction of both spi2a mRNA and protein by a combination of IFN-γ and bacterial products.

Identification of spi2a-ISG15 conjugates in activated macrophages

While studying the expression of spi2a protein in the RAW264.7 macrophage cell line, we observed that higher molecular mass forms of spi2a were detected (Fig. 3*A*). An N-terminally HA-tagged version of spi2a (spi2a-HA) was transiently expressed in the RAW264.7 macrophage cell line, cytoplasmic extracts were separated by SDS-PAGE, and spi2a was detected with a mAb to the HA tag. The majority of the spi2a-HA was present in a band with the electrophoretic mobility predicted for the tagged protein, 50 kDa (Fig. 3*A*). Slower migrating forms of ~65 and 80 kDa were also detected. The 42-kDa band corresponds to a spi2a-HA degradation product.

To identify the protein components of the 65- and 80-kDa complexes, we purified spi2a from transiently transfected RAW264.7 cells using a version of spi2a with both an N-terminal HA and a six-histidine tag (spi2a-HisHA). Spi2a-HisHA was first affinity purified from transiently transfected RAW264.7 cells with Sepharose beads conjugated to a mAb to the HA tag, eluted, and then affinity purified with Ni^{2+} beads that bind the His tag. The protein was eluted from the Ni^{2+} beads, separated by SDS-PAGE, and silver stained (Fig. 3*B*). Two of the silver-stained bands that matched precisely with the 65- and 80-kDa forms of spi2a detected by Western blotting (Fig. 3*B*, arrows) were cut out, and the protein content was identified by tandem mass spectrometry. The 80-kDa band contained only two proteins, spi2a and murine ISG15, also known as ubiquitin cross-reactive protein; Fig. 3*C* shows the sequence of ISG15 with the three independently identified peptides boxed. The ISG15 peptides identified covered 30.6% of the protein sequence. The 65-kDa band also contained spi2a and ISG15, as well as an apparent contaminant, TCP-1γ, a 66-kDa chaperone.

ISG15 is a 15-kDa protein that contains two ubiquitin homology domains. It has been shown to conjugate to cellular proteins in a process analogous to that for ubiquitin, using homologous, but distinct, enzymes (22–24). Thus, the 65-kDa form of spi2a likely represents spi2a (50 kDa) covalently bound to one molecule of ISG15, whereas the 80-kDa form represents spi2a bound to two molecules of ISG15. The other bands seen on the silver-stained gel contained serum proteins, including serum albumin (69 kDa) and α_2-macroglobulin (165 kDa), and were most likely derived from the FCS in the tissue culture medium.

To confirm that the spi2a 65 and 80 kDa bands contained ISG15, we affinity purified spi2a-HisHA from RAW264.7 cells and probed Western blots with Abs to the HA tag or to endogenous ISG15. Polyclonal Abs raised against purified human ISG15 identified the 65-kDa and 80-kDa bands that also reacted with the anti-HA Ab (Fig. 4) but did not label the 50-kDa band that contained the unconjugated spi2a.

We next coexpressed spi2a-HA and V5-tagged ISG15 (ISG15-V5) in RAW264.7 cells and assayed for the presence of ISG15-V5-spi2a conjugates. The 65- and 80-kDa spi2a conjugates were detected when spi2a-HA was transfected alone or cotransfected with the ISG15-V5 construct (Fig. 5, *top*). As expected, the conjugates were slightly larger in the cells cotransfected with the ISG15 containing the 14-aa V5 tag. A replicate blot was probed with Abs to the V5 tag present on ISG15. When ISG15-V5 was transfected alone, we detected a ladder of bands that presumably corresponds to ISG15 conjugated to a variety of cellular proteins, including spi2a (Fig. 5, *middle*). When spi2a-HA and ISG15-V5 were coexpressed, ISG15 was found in four principal bands, two

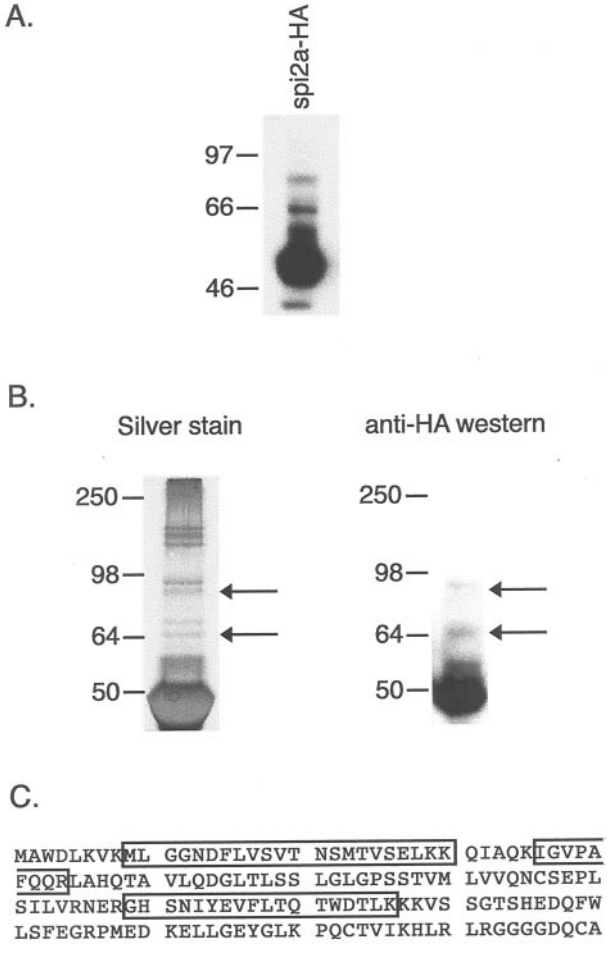

FIGURE 3. spi2a is present in higher molecular mass conjugates containing ISG15 in RAW264.7 macrophages. *A*, Cytoplasmic extract from RAW264.7 cells transiently transfected with an expression construct for spi2a-HA was analyzed by Western blot after separation by SDS-PAGE. spi2a expression was detected using a mAb to the HA tag. *B*, spi2a protein was affinity purified from RAW264.7 cells transiently transfected with spi2a-HisHA using Sepharose beads conjugated to an anti-HA mAb followed by Ni^{2+} beads to bind the six-His tag. The eluate from the Ni^{2+} beads was separated by SDS-PAGE and either silver stained (*left*) or Western blotted (*right*) with detection by the anti-HA Ab. The two bands designated with arrows were identified by both Western blot and silver stain. The two bands running at 65 and 80 kDa (arrows) were cut out of the gel, digested with trypsin, and subjected to tandem mass spectrometry to determine the peptide sequence. *C*, Amino acid sequence of ISG15 with the three tryptic peptides identified by mass spectrometry of the 80-kDa band (upper arrow, *B*) shown in the boxes.

of which corresponded to the spi2a complexes detected with the HA Ab. It is striking that overexpression of spi2a along with ISG15 caused the redistribution of ISG15 into four bands as opposed to the numerous bands seen when ISG15-V5 was expressed alone. This may reflect a rate-limiting step in the conjugation process exposed by overexpression of both proteins. The lower panel in Fig. 5 shows that unconjugated ISG15-V5 was not limiting regardless of whether spi2a was overexpressed.

ISG15 expression is induced during macrophage activation

Macrophages are strongly activated by bacterial DNA, and transient transfection using bacterial vectors results in the enhanced expression of many proteins associated with the activated state (25). We therefore examined the conjugation of spi2a and ISG15 in stably transfected macrophages, where the transient activation induced by the bacterial plasmid DNA has subsided. Surprisingly, stable clones of RAW264.7 cells expressing spi2a-HA did not contain the 65- and 80-kDa spi2a-ISG15 conjugates that were present in transiently transfected cells (Fig. 6*A*). However, LPS induced these conjugates in the macrophages (Fig. 6*A*), implying that LPS had induced the expression of ISG15, which could now conjugate with stably transfected spi2a. This was confirmed by Western blot analysis demonstrating that ISG15 was essentially undetectable in untreated RAW264.7 cells and was very strongly induced after LPS stimulation (Fig. 6*B*). The hypothesis that bacterial DNA was inducing ISG15 during transient transfections was similarly confirmed (Fig. 6*B*).

Although the RAW 264.7 cell line is useful for transfection studies, it was important to demonstrate the regulation of ISG15 in primary macrophages, both in vitro and in vivo. LPS induced ISG15 in resident peritoneal macrophages; the protein was detected within 1 h and reached maximal levels of expression 4 h after stimulation with LPS (Fig. 7*A*). LPS also induced the expression of spi2a in resident peritoneal macrophages with similar kinetics to ISG15. Interestingly, although IFN-γ alone strongly induced spi2a, it had no effect on the expression of ISG15 (Fig. 7*A*), demonstrating that the regulation of these two proteins could be uncoupled.

We identified spi2a as a protein induced in response to macrophage activation by BCG infection in vivo (Fig. 1), and we therefore investigated whether ISG15 is also induced under these conditions. Infection of mice i.p. with BCG resulted in the very strong induction of both spi2a and ISG15 in macrophages in vivo (Fig. 7*B*). This demonstrates that the induction of ISG15 and spi2a in vitro accurately reflects the more complex process of macrophage activation during bacterial infection in vivo.

Discussion

Using cDNA array analysis, we have identified spi2a as a protein that is induced in macrophages activated during infection with intracellular bacteria. The cDNA for spi2a was originally cloned from a chondrocyte cell line (6) and subsequently demonstrated to be expressed in hemopoietic progenitor cells (7). Interestingly, Hampson et al. (7) found spi2a mRNA to be down-regulated during granulocyte macrophage differentiation of a multipotential hemopoietic progenitor cell line and during macrophage differentiation of bipotential granulocyte/macrophage precursor cells isolated from mouse bone marrow. They found that spi2a mRNA was absent from mature macrophages differentiated from the progenitor cell line, which is consistent with our results showing that spi2a mRNA and protein are undetectable in unstimulated resident peritoneal macrophages.

Up-regulation of spi2a appears to be a general response of macrophages to bacterial infection both in vivo and in vitro. In addition to BCG, both Gram-positive (*L. monocytogenes*) and Gram-negative (*S. typhimurium*) infections in vivo induced macrophages that had up-regulated spi2a mRNA. These bacteria, along with *M. bovis* BCG, used to initially identify spi2a, are all intracellular pathogens that can live within macrophages in the infected host. Extracellular pathogens, including yeast (zymosan) as well as the Gram-positive bacterium *Staphylococcus aureus*, were also able to activate the spi2a promoter in vitro in RAW264.7 macrophages. Indeed, the spi2a promoter was induced by a variety of pathogens, and their products, that are known to activate macrophages for production of proteins important in the antimicrobial immune response, such as TNF-α and IL-12. This suggests that induction of spi2a is a general response of macrophages to infection with pathogens and is not specific to mycobacterial infections.

spi2a mRNA and protein are induced in vitro not only by bacterial products but also by the cytokine IFN-γ. This cytokine, produced by activated natural killer cells and T cells, is critical for a

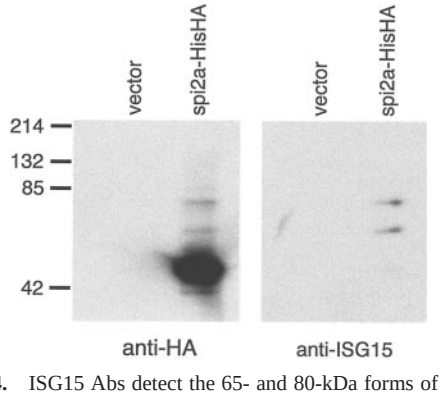

FIGURE 4. ISG15 Abs detect the 65- and 80-kDa forms of spi2a. Protein from RAW264.7 cells transiently transfected with vector alone or vector encoding spi2a-HisHA was affinity purified as in Fig. 3 and separated by SDS-PAGE; Western blots were probed with affinity-purified polyclonal serum to ISG15 (*right*). This blot was then stripped and reprobed with an anti-HA mAb (*left*).

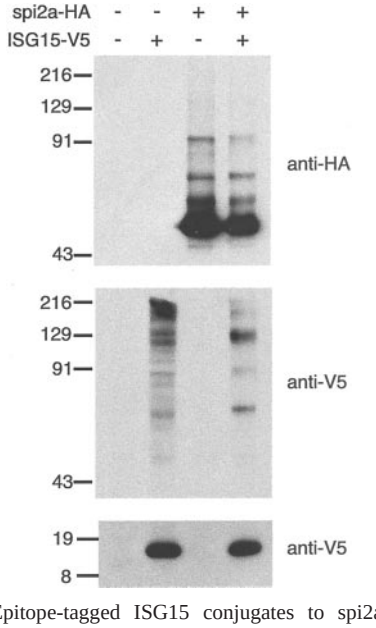

FIGURE 5. Epitope-tagged ISG15 conjugates to spi2a. RAW264.7 cells were transiently transfected with vector alone, vector encoding spi2a-HA, and/or ISG15-V5. Cytoplasmic extracts were separated by SDS-PAGE, and Western blots were probed with anti-HA (*top*) or anti-V5 (*middle* and *bottom*) mAbs. The bottom panel is a shorter run of the middle panel to show free ISG15-V5 protein.

successful immune response to intracellular pathogens, including those used in this study (2, 26). During activation of macrophages, IFN-γ is essential for the induction of bactericidal mechanisms including stimulation of reactive oxygen intermediate production and induction of iNOS for the generation of reactive nitrogen intermediate (3, 27, 28). IFN-γ also induces the expression of class II MHC molecules that allow activated macrophages to present Ag to CD4 T cells (2, 27). Pretreatment of macrophages with IFN-γ sensitized them for induction of the spi2a promoter by bacterial products and for the production of spi2a protein by LPS. This activation of the spi2a promoter by IFN-γ and bacteria, or their products, is clearly more than additive. This is reminiscent of the induction of iNOS and the IL-12 p40 subunit in macrophages; both are slightly induced by LPS, but the combination of LPS with IFN-γ potently induces these proteins (29–32). The high levels of spi2a mRNA and protein in macrophages from BCG-infected mice may be due to the fact that both IFN-γ and bacteria and their products are available to activate macrophages in this setting.

Both bacterial products and IFN-γ regulated spi2a at the transcriptional level. The induction of the spi2a promoter by LPS and other bacterial products is consistent with the presence of a consensus NF-κB binding site in the promoter region (−259 to +46) used to drive the luciferase reporter in these experiments. Hampson et al. (18) found that this NF-κB binding site was critical for maximal induction of the spi2a promoter in a multipotential hemopoietic progenitor cell line and in primary murine splenocytes, presumably measuring expression in T cells. NF-κB translocation to the nucleus is a well-documented consequence of macrophage activation through Toll-like receptors, which have been shown to signal downstream of bacteria and their products (33). Interestingly, the spi2a promoter used in these studies also contains a predicted STAT binding site consensus sequence at bases −128 to −137 (J. A. Hamerman, L. Hampson, and A. Aderem, unpublished observations). This STAT binding site may explain the responsiveness of the spi2a promoter to IFN-γ. spi2a can be added to the list of other IFN-γ-responsive genes important in macrophage function during infection.

The role of spi2a in activated macrophages is not yet clear. Serpins are a protein superfamily with conserved structure and have been demonstrated to regulate serine and cysteine protease function both extracellularly and intracellularly (8). Serpins have been shown to participate in diverse processes mediated by proteases including complement activation, coagulation, fibrinolysis, extracellular matrix degradation, and apoptosis (9). Although some members of the serpin family, such as OVA and angiotensin, are not functional protease inhibitors (8), spi2a possesses the serpin proximal hinge motif indicative of a functional inhibitor (34) (J. A. Hamerman and A. Aderem, unpublished observations). We therefore propose that spi2a is a functional protease inhibitor that regulates protease activity in activated macrophages and that this activity is involved in the function of macrophages during infection with intracellular bacteria. Interestingly, spi2a is up-regulated in activated $CD8^+$ T cells as well as activated macrophages (7) and therefore during infection may be expressed in both cell types. spi2a may play a similar role in both or may regulate different proteases in the each cell type.

Although the majority of well-characterized serpins are secreted, some function intracellularly (8). spi2a is primarily expressed intracellularly by several criteria. spi2a lacks an N-terminal signal sequence, it shows diffuse cytoplasmic staining by immunofluorescence in LPS-treated resident peritoneal macrophages, and it cannot be detected in supernatants from LPS-treated resident peritoneal macrophages or stably transfected RAW264.7 cells, whereas cell lysates are strongly positive (J. A. Hamerman, L. A. Schroeder, and A. Aderem, unpublished observations). Several cytoplasmic serpins have been shown to regulate apoptosis, a process dependent on proteolytic cascades. This includes inhibition of caspase 1 by the cowpox virus serpin crmA (35) and the inhibition of granzyme B by the human serpin PI-9 (36). Other

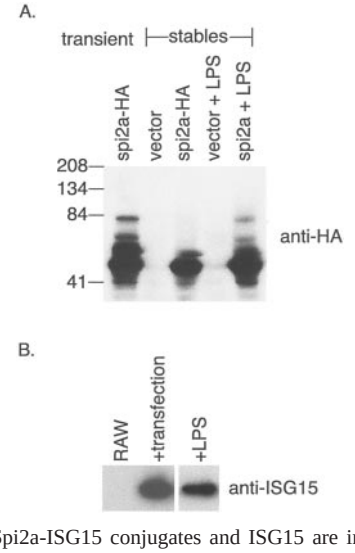

FIGURE 6. Spi2a-ISG15 conjugates and ISG15 are induced by transfection and LPS treatment. *A*, Cytoplasmic extracts from RAW264.7 cells either transiently transfected with the spi2a-HA construct or stable clones made with vector alone or the spi2a-HA construct were analyzed as in Fig. 1*A*. The stable clones were either untreated or treated overnight with 100 ng/ml LPS. *B*, Cytoplasmic extracts from RAW264.7 cells that were untreated, transiently transfected with empty vector (+transfection), or treated with 100 ng/ml LPS overnight were analyzed by Western blot with affinity-purified polyclonal Abs to ISG15.

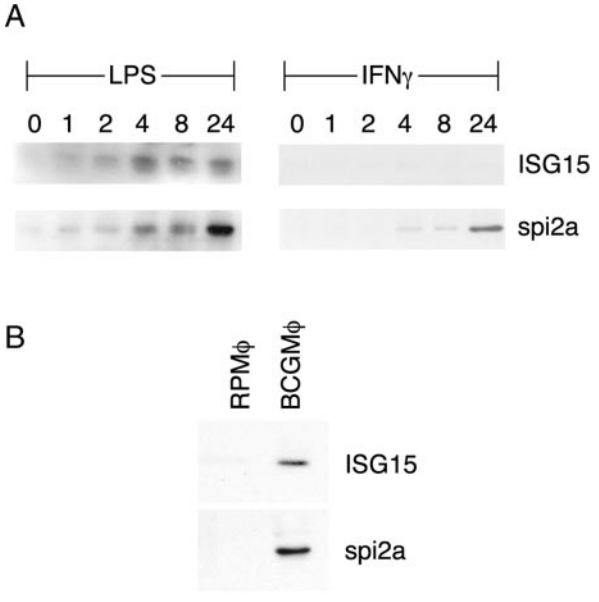

FIGURE 7. ISG15 and spi2a are induced in in vitro and in vivo activated primary murine macrophages. *A*, Cytoplasmic extracts were generated from resident peritoneal macrophages that were treated with 100 ng/ml LPS or 10 U/ml IFN-γ from 0 to 24 h as indicated. ISG15 was detected as in Fig. 4 and spi2a was detected as in Fig. 1B. *B*, Cytoplasmic extracts from peritoneal macrophages from uninfected mice (RPMϕ) or mice infected 12 days previously with BCG (BCGϕ) were analyzed as in *A*.

cytoplasmic serpins have been shown to protect cells from their own proteases. PI-6 has been shown to inhibit cathepsin G, a neutrophil granule protease (37). Presumably, the cytoplasmic PI-6 protects the neutrophil from granule rupture before release from the cell. PI-9, described above, is expressed by cytotoxic T cells and NK cells, which also express the PI-9 target, granzyme B, in their cytotoxic granules (36). It has been proposed by Bird et al. (38) that PI-9 protects T cells and NK cells against misdirected granzyme B after degranulation or leakage of granzyme B from cytotoxic granules within the cell. spi2a may have functions analogous to those of these well-characterized serpins. Macrophages have an extensive lysosomal system containing a variety of proteases that are up-regulated in response to IFN-γ treatment (39–41). This enables the macrophage to degrade bacteria and other ingested pathogens once they have been killed. The potential release of lysosomal enzymes into the cytoplasm could result in macrophage cell death, and therefore a mechanism involving spi2a may exist to protect against this risk.

We have also demonstrated that spi2a forms conjugates with ISG15, a ubiquitin homolog. Two complexes are detected, 65- and 80-kDa conjugates. This suggests that the 50-kDa spi2a is complexed with either one or two molecules of the 15-kDa ISG15. ISG15 is a member of a small family of proteins that demonstrate significant sequence similarity to ubiquitin and that covalently modify other cellular proteins (42). It contains two ubiquitin-like domains with 43 and 62% homology to ubiquitin (10, 14). The mechanism of conjugation of ISG15 to cellular substrates has been proposed to be analogous to that for ubiquitin involving homologous but not identical enzymes (22, 23, 43, 44). Ubiquitin can form polyubiquitin chains from one lysine residue in a target protein, and it is possible that the spi2a-ISG15 complex with two ISG15 molecules is the result of a di-ISG15 chain. Alternatively, this 80-kDa complex may reflect conjugation of a single ISG15 molecule to two distinct sites on spi2a. The pattern of ISG15 conjugates within cells is distinct from that of ubiquitin-modified proteins, suggesting that ubiquitin and ISG15 have different target proteins (14). Although many protein targets have been described for ubiquitin conjugation, spi2a is the first ISG15-modified protein to be reported.

ISG15 and spi2a are both induced during in vitro and in vivo macrophage activation, although their induction in this process can be uncoupled. Thus, whereas spi2a is profoundly induced by either LPS or IFN-γ, ISG15 is induced only by bacterial products such as LPS or bacterial DNA, which both signal through Toll-like receptors (33). ISG15 conjugation to spi2a correlates with induction of these two proteins in activated macrophages. Previously, ISG15 had been shown to be induced by type 1 IFNs, and this induction was attributed to the presence of an IFN-stimulated response element in the ISG15 promoter (45). Interestingly, LPS induces not only ISG15 expression but also production of IFN-β from macrophages (46). It is therefore possible that LPS-induced IFN-β acts in an autocrine manner on macrophages resulting in ISG15 production, although LPS could also induce ISG15 directly. However, we demonstrate here that ISG15 protein can be detected in resident peritoneal macrophages after 1 h of LPS treatment, suggesting that the effect of LPS is most likely direct. This does not preclude that the higher levels seen at later times are not due, in part, to autocrine IFN-β production.

The functional consequences of spi2a conjugation to ISG15 are unclear. Although ubiquitin modification targets proteins for degradation via the proteasome, there is no evidence that ISG15 has this function. Indeed, treatment of spi2a-transfected RAW264.7 cells with proteasome inhibitors had no effect on the accumulation of spi2a-ISG15 conjugates (J. A. Hamerman, L. A. Schroeder and A. Aderem, unpublished observations), whereas this accumulates ubiquitin-protein conjugates (47, 48). These conditions have also been shown to have no effect on the half-life of total ISG15 conjugates in a lung carcinoma cell line while producing significant effects on total protein degradation (J. Narasimhan and A. Haas, unpublished observations). Loeb and Haas (24) have reported that ISG15 conjugates colocalize with intermediate filaments in a lung carcinoma cell line, but we have not been able to see this in resident peritoneal macrophages or in RAW264.7 cells (J. A. Hamerman, L. A. Schroeder, and A. Aderem, unpublished observations). We have also not detected spi2a colocalized with intermediate filaments in these cells. Despite this negative result, it is known that LPS regulates intermediate filaments. It induces the reorganization of the vimentin network into bundles in microglia and fibroblasts (49, 50), whereas IFN-β, which is induced by LPS treatment, stimulates transcription of the vimentin gene in epithelial cells (51). ISG15 conjugation to spi2a may target spi2a to intermediate filaments allowing for regulation of protease activity at this site by this serpin. Interestingly, it has recently been shown that the influenza B virus NS1 protein inhibits conjugation of ISG15 to cellular proteins (44). This suggests that ISG15 conjugation is an effective part of the host response to viral infection, because pathogens often target pathways that decrease their ability to survive and replicate.

In summary, we have identified both spi2a and ISG15 as proteins which are induced in activated macrophages and that physically interact in these cells. spi2a expression is up-regulated by interactions with a variety of bacterial pathogens and their products as well as by IFN-γ, an abundant cytokine during bacterial infection; whereas ISG15 is induced by bacterial products, but not IFN-γ. This is the first demonstration of a target for ISG15 conjugation, and its identification will help advance the understanding of both the biochemical mechanism by which ISG15 conjugation occurs and the functional consequences of ISG15 modification of cellular proteins.

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