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Aging Enhances the Production of Reactive Oxygen Species and Bactericidal Activity in Peritoneal Macrophages by Upregulating Classical Activation Pathways

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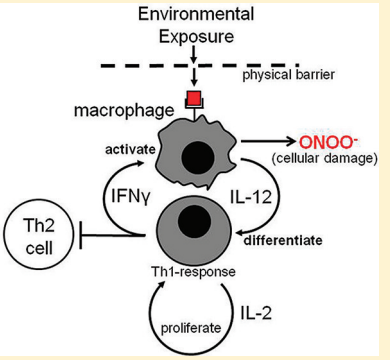
Supporting Information

ABSTRACT: Maintenance of macrophages in their basal state and their rapid activation in response to pathogen detection are central to the innate immune system, acting to limit nonspecific oxidative damage and promote pathogen killing following infection. To identify possible age-related alterations in macrophage function, we have assayed the function of peritoneal macrophages from young (3−4 months) and aged (14−15 months) Balb/c mice. In agreement with prior suggestions, we observe age-dependent increases in the extent of recruitment of macrophages into the peritoneum, as well as ex vivo functional changes involving enhanced nitric oxide production under resting conditions that contribute to a reduction in the time needed for full activation of senescent macrophages following exposure to lipopolysaccharides (LPS). Further, we observe enhanced bactericidal activity following *Salmonella* uptake by macrophages isolated from aged Balb/c mice in comparison with those isolated from young animals. Pathways responsible for observed phenotypic changes were interrogated using tandem mass spectrometry, which identified age-dependent increases in levels of proteins linked to immune cell pathways under basal conditions and following LPS activation. Immune pathways upregulated in macrophages isolated from aged mice include proteins critical to the formation of the immunoproteasome. Detection of these latter proteins is dramatically enhanced following LPS exposure for macrophages isolated from aged animals; in comparison, the identification of immunoproteasome subunits is insensitive to LPS exposure for macrophages isolated from young animals. Consistent with observed global changes in the proteome, quantitative proteomic measurements indicate that there are age-dependent abundance changes involving specific proteins linked to immune cell function under basal conditions. LPS exposure selectively increases the levels of many proteins involved in immune cell function in aged Balb/c mice. Collectively, these results indicate that macrophages isolated from old mice are in a preactivated state that enhances their sensitivities to LPS exposure. The hyper-responsive activation of macrophages in aged animals may act to minimize infection by general bacterial threats that arise due to age-dependent declines in adaptive immunity. However, this hypersensitivity and the associated increase in the level of formation of reactive oxygen species are likely to contribute to observed age-dependent increases in the level of oxidative damage that underlie many diseases of the elderly.



Within the animal kingdom, the innate immune response is highly conserved. Specific classes of immune cells recognize characteristic pathogen-associated biomolecules [e.g., conserved cell wall components like lipopolysaccharides (LPS)] to orchestrate their rapid clearance at localized sites. For example, macrophages rapidly engulf and kill entrapped pathogens through a coordinated oxidative burst, simultaneously releasing inflammatory cytokines (e.g., TNFα) to recruit additional immune cells to the site of infection. In vertebrates, activated macrophages also act as an interface with the adaptive immune system through the presentation of antigenic determinants derived from engulfed pathogens that act to activate and maintain T-cell activation following their recruitment to sites of infection. Microbial infections, in turn, reprogram cytotoxic lymphocytes and T-helper cell responses as part of the adaptive immune system to induce their proliferation and promote the release of IFNγ to sensitize macrophages at distant sites. IFNγ exposure acts to promote macrophage activation upon bacterial recognition to upregulate antigen presentation, the release of inflammatory cytokines, and the production of reactive oxygen species (ROS) that act to kill microorganisms. Control of macrophage activation is critical as the oxidative burst, resulting from their activation, inflicts collateral damage to host macromolecules and tissues, which contributes to a range of different age-related diseases.[1,2]

Common models of macrophage aging emphasize the accumulation of oxidative DNA damage that results in their functional dysregulation, reported to impair the ability to respond to LPS, generate ROS, and present antigens through class I and class II major histocompatibilty complex (MHC-I and MHC-II, respectively) pathways necessary for the activation of cytotoxic lymphocytes and T-helper cells.[3] Alternatively, changes in levels of circulating cytokines may induce differentiation to alter macrophage polarity, acting to

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modify the repertoire and magnitude of available functional responses. Complicating an understanding of age-dependent declines in immune function is the coupling between the innate and adaptive immune systems, which act together to coordinate cellular responses to pathogen exposure. While it is understood that adaptive immunity declines with age,[4,5] discrepant results have been reported regarding age-dependent changes in macrophage function and their importance in downgrading immune defenses, which predispose aged animals to infection and contribute to the development of many of the diseases of the elderly.[6−8] For example, isolated resident peritoneal macrophages from middle-aged (12 months) and old (21 months) mice have been reported to show an enhanced ability to kill herpes simplex virus in comparison to young (2 months) controls, despite reported decreases in their ability to generate nitric oxide and other reactive oxygen species (ROS) known to mediate bacterial killing.[9] Similar increases in pathogen clearance are observed for aged Balb/c mice (18 months) when animals were challenged with a bacterial infection involving *Leishmania major*, despite the absence of any significant differences in rates of phagocytosis, bacterial killing, or nitric oxide production following isolation and uniform sensitization of peritoneal macrophages by IFNγ.[10] An important clue to these apparently contradictory results comes from the observation that enhanced rates of bacterial killing observed in aged mice depend on their prior exposure to normal circulating pathogens, because housing under clean room conditions abrogates age-dependent increases in bacterial resistance.[10] These latter results suggest that age-dependent alterations in macrophage function result from an enhanced sensitivity to environmental exposure, which may occur due to age-dependent reductions in physical barriers that limit pathogen entry and act to sensitize macrophages to promote their rapid activation in response to infection.

As environmentally induced changes in macrophage function should result in characteristic alterations in the proteome that are indicative of shifts in activation pathways, we have examined possible linkages between protein abundance changes and molecular pathways involving immune function, and their relationship to phenotypic changes in macrophage function. Peritoneal macrophages were isolated from young (3−4 months) and aged (14−15 months) Balb/c mice, which represents a normal model for use in aging measurements.[9,10] The aged mice (14−15 months) used in our measurements are near the average lifespan of this mouse strain, which is 485 ± 9 days (~16 months),[11] permitting an investigation of age-dependent cellular changes prior to the onset of late-life pathologies that can complicate mechanistic interpretations. Macrophage functions were assayed using simple in vivo measurements that indicate age-dependent increases in the abundance of macrophages in the peritoneum in response to elicitation by an irritant injected into the peritoneum that is indicative of increased motility and macrophage activation. Mechanistic linkages between observed phenotypic changes in macrophage function and molecular pathways involving immune function were identified using mass spectrometry-based measurements of protein abundance changes, which involved a shotgun proteomic analysis in which tandem mass spectrometry was used to identify 1847 proteins with annotated functions for subsequent quantitation of protein abundance changes using the accurate mass and elution time (AMT) tag proteomic strategy. Consistent with observed functional changes and hypotheses developed using tandem MS data, we observe an age-dependent upregulation of specific immune cell pathways (e.g., antigen presentation) and abundance changes of 77 proteins linked to macrophage activation. There is no indication of any impairment of normal cellular pathways involving macrophage activation in aged animals; rather, these results are indicative of an age-dependent upregulation of normal classical activation pathways.

EXPERIMENTAL PROCEDURES

Materials. Male Balb/c mice (3−4 and 14−15 months) were from Charles River Laboratories International Inc. (Wilmington, MA). Bio-Gel P-100 polyacrylamide beads (45−90 μm) were from Bio-Rad (Richmond, CA). Bacterial LPS from *Escherichia coli* strain O127:B8 was from Sigma Chemical (St. Louis, MO). Interferon was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Ampicillin, RPMI 1640 medium (0030078DJ), fetal bovine serum (FBS), penicillin, and streptomycin were from Gibco (Carlsbad, CA). FITC-labeled anti-mouse F4/80 macrophage specific antibodies were from eBioscience, Inc. (San Diego, CA). Calcein acetoxymethyl (AM) ester and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) were from Invitrogen (Carlsbad, CA).

Macrophage Elicitation. When indicated, macrophages were elicited by injection (1 mL) of a 2% (v/v) sterile solution of washed and hydrated polyacrylamide beads in phosphate-buffered saline (PBS) into the peritoneal cavity 5 days prior to their isolation, as previously described.[12] Our experimental design is in accordance with all prior reports that address age-dependent changes in macrophage function, which commonly isolate peritoneal macrophages following elicitation at a single time point 3−5 days following the introduction of an irritant (in our case Bio-Gel polyacrylamide beads).[8,13−20] As described by Melnicoff and co-workers,[21] homogeneous populations of peritoneal macrophages are isolated within this time window to prevent contamination by neutrophils and resident macrophages that becomes problematic when macrophages are collected at longer times following elicitation.

Macrophage Isolation. Resident or elicited peritoneal macrophages were isolated using standard peritoneal lavage procedures following asphyxiation using CO_2.[12] Briefly, PBS (10 mL) was injected into the caudal half of the peritoneal cavity using a 25-gauge needle; whole mice were gently rocked for 10 s, and peritoneal cells were slowly withdrawn using a 19-gauge needle and collected into a conical tube on ice. Isolated cells were plated in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (10%, v/v), penicillin (1%, v/v), and ampicillin (1%, v/v) at a constant cell density (1×10^6 cells/p100 plate) and incubated for 1 h at 37 °C. Nonadherent cells were removed by washing the plates five times in warm PBS (500 μL), and remaining adherent macrophages were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C overnight prior to treatment and harvest. The purity and viability of isolated cells were analyzed using an Agilent 2100 Bioanalyzer Microfluidics platform for flow cytometry with two-color analysis of fluorescently stained cells and standard immunohistochemistry methods using a Nikon Eclipse TE200 epifluorescence microscope equipped with Metamorph imaging software to quantify the correspondence between multiple fluorescence signatures. Macrophage purity was determined to be greater that 95% of isolated cells through a comparison of FITC-labeled anti-mouse F4/80 macrophage specific antibodies to identify cells in comparison to total cells measured using DAPI labeling of double-stranded

DNA. Viability was measured by comparing fluorescence signals associated with cell esterase activity visible upon cleavage of calcein AM in comparison to those for FITC-labeled anti-mouse F4/80 macrophage specific antibodies.

Nitric Oxide Measurements. Time-dependent increases in the levels of nitric oxide were measured by following the accumulation of the stable nitrite end product using the Griess reagent (Pierce Inc., Rockford, IL). Prior to measurement, all nitrate was enzymatically converted to nitrite using nitrate reductase. Nitrite concentrations in conditioned media were determined on the basis of standard curves calibrated using sodium nitrite as a standard, as we have previously described.[22]

Bactericidal Activity. Primary peritoneal or RAW 264.7 macrophages were challenged with *Salmonella typhimurium* at a multiplicity of infection of 100, essentially as previously described.[23−25] Briefly, *S. typhimurium* 14028 cells were cultured the day before the assay in LB broth at 37 °C overnight and were harvested by centrifugation (12000 rpm for 1 min) before resuspension at a final density of 5×10^5 cells/mL in 24-well cell-culture plates (i.e., 500000 macrophages/well). Prior to bacterial challenge, macrophages were treated with 100 units/mL interferon γ (IFNγ) and rinsed, and the prepared *S. typhimurium*-laden medium were directly added to the plate of macrophages.[26] The infection proceeded as plates were placed back into the incubator under standard conditions (37 °C in 5% CO_2). Following incubation for 30 min to permit macrophages to phagocytize the *S. typhimurium*, extracellular bacteria were rinsed off with PBS. Fresh medium (12.5 μg of G418/mL) replaced the bacteria-laden medium to eliminate extracellular bacteria and prevent the extracellular replication and invasion of any *S. typhimurium* remaining on the plate. Macrophages infected with *S. typhimurium* were then further incubated, and at indicated times, cells were washed and lysed [PBS, 1% Triton X-100, and 0.1% sodium dodecyl sulfate (SDS) for 5 min at room temperature]. Remaining viable bacteria were detected as colonies following the serial dilution of the cellular lysates onto LB agarose plates that were incubated overnight at 37 °C.

Macrophage Lysis. Following removal of media, cells were rinsed once with chilled D-PBS (Invitrogen) and incubated in chilled 20 mM Tris (pH 8.0), 1% Nonidet P-40, 0.15 M NaCl, 1 mM Na_2PO_4, and 1 mM EGTA. Cell lifters were used to manually scrape and transfer cells into a chilled glass homogenizer, and following cell disruption (a 10-stroke homogenization), lysates were immediately centrifuged for 30 min at 9300*g* and 4 °C. The supernatant was removed and stored at −80 °C. Cellular disruption in the presence of detergent permits quantitative measurements of total lysates to be evaluated as membrane disruption and protein solubilization enhance the overall proteomic coverage. In the absence of protein solubilization, membrane-associated proteins are lost from the sample during sample processing. As many of the key proteins associated with macrophage activity are linked to membrane processes, it is critical to include a solubilization step for accurate assessments of proteomic changes. Lysates were subjected to low-speed centrifugation (9300*g* on a table-top centrifuge) prior to analysis to prevent any large particles from clogging the LC equipment, and there was no detectable loss of protein following this procedure.

Cysteine Alkylation and Trypsin Digestion. Lysates were dialyzed against ammonium bicarbonate prior to addition of 8 M urea to denature proteins. Cysteine residues in lysates were reduced using tris(2-carboxyethyl)phosphine (TCEP) (Bond Breaker TCEP solution) (5 mM) (Thermo Scientific, Rockford, IL), alkylated using iodoacetamide (25 mM), and sonicated in a UTR200 sonoractor (Hischler, Teltow, Germany) at 50% intensity for 3 min, as described previously.[27] Following a 10-fold dilution of samples into a freshly prepared 50 mM ammonium bicarbonate solution (pH 7.8) and 1 mM $CaCl_2$, samples were digested using trypsin (1:50, w/w ratio of trypsin to sample protein) for 1 min in a Barocycler NEP-3229 instrument, as previously described.[28] Each digest was desalted using Supelco (St. Louis, MO) Supelclean C-18 tubes, as described previously,[29] and concentrated (1 mg/mL) using vacuum centrifugation.

LC−MS Analysis and Peptide Identification. A quantitative analysis of changes in the cellular proteome involves a two-step process. First, in stage 1, tandem MS (MS/MS) measurements are taken following proteolytic digestion and strong cation exchange (SCX) fractionation of proteins from peritoneal macrophage lysates obtained from young and aged animals into 30 liquid chromatography (LC) fractions for analysis using a linear trap quadrupole (LTQ) ion-trap mass spectrometer (ThermoElectron Corp., San Jose, CA), as described previously.[30] The MS/MS spectra were analyzed using SEQUEST Bioworks version 3[31] against an IPI *Mus musculus* database downloaded from the National Center for Biotechnology Information (i.e., M_Musculus_2006-07-25_IPI, which contains 94146 entries). The mass tolerances for the precursor and fragment ions were 2.5 and 0.8 Da, respectively. Database search parameters included a dynamic modification of 16 Da for Met oxidation and a fixed modification of 57 Da for Cys carbamylation. Only peptides with charges of +2 and +3 were analyzed for fragmentation and SEQUEST analysis. Confident peptide identification with a false discovery rate of 1% (i.e., $q < 0.01$) involved fitting the data to a sum of Gaussian distributions following correction of X_{corr} values to take into account peptide length, where corrected $X_{corr} = \ln(X_{corr})/\ln(\text{peptide length})$ and corrected $\Delta C_n = (\Delta C_n)^{1/2}$.[27,32] Sequences, charge states, masses, and peptide identification scores obtained from the SEQUEST algorithm for all identified peptides are included as Supporting Information. No upper limit was prescribed for the number of missed or nonspecific peptide cleavages. However, as tabulated in the Supporting Information, the vast majority (>92%) of identified peptides contain two tryptic cleavage sites, providing enhanced support for the idea that the SEQUEST algorithm combined with the mass resolution of our instrumentation provides accurate peptide assignments. We find that 6% of the identified peptides contain a single tryptic cleavage site. Peptides identified from these tandem MS measurements were used to build an accurate mass and elution time (AMT) database that matches the masses of individual peptide sequences and normalized elution times to permit unique peptide identifications, as previously described.[33] The AMT database is necessary for all quantitative changes in the abundances of individual proteins.

Quantitative Measurements of Protein Abundance Changes. Using the AMT database, in stage 2 we use high-resolution reversed phase capillary chromatography coupled to high-resolution LTQ-Orbitrap XL MS to quantify changes in the abundance of individual peptides from a consideration of the ratio of ion currents for individual AMT tags, as previously described.[34,35] LC−MS spectra are first processed to detect charge and isotopic masses in individual mass spectra using Decon2LS (http://ncrr.pnl.gov/software/), and identified spectra are further processed using the VIPER program to

calibrate elution times, refine the mass calculation, and match the LC−MS features to AMT tags in the reference database.[36] In these latter measurements, the grouped features for each identified peptide are represented by the median value obtained across three LC−MS runs. These data were loaded into the software tool DAnTE for quantitative data analysis, which allows a direct comparison of identified peptides across data sets for analysis and visualization of abundance differences.[37] Peptide abundances were first log base 2 transformed, and an outlier check was applied by observing the Pearson correlations between data sets. Any prominent outlier data set that had weak correlations (<0.7) was excluded from further analysis, as previously described.[38] A linear regression-based normalization method (available in DAnTE) was applied next, within each replicate category. The central tendency adjusted peptide abundances were used to infer the corresponding protein abundances via the Rrollup algorithm in DAnTE.[37] This tool permitted a determination of protein abundance changes, where the most abundant peptide across all data sets is used as a reference to calculate the ratios of protein abundances. During the Rrollup step, the Grubbs outlier test was applied with a p value cutoff of 0.05 to further remove any outlying peptides. Finally, a t test identified significant abundance differences, using a p value of <0.05. Protein abundances are the median values of the resulting peptide abundances, where statistical significance is calculated from the ratio of the protein abundances using a cutoff at 5% FDR. IPI protein identifiers obtained from the individual proteins were used for data mining and retrieval using internal cross identifier mappings (i.e., mapping form protein to gene identifiers) and further pathway and functional enrichment data retrieval in conjunction with the Bioinformatics Resource Manager (BRM) software.[39] Estimates of total proteome coverage utilized David Bioinformatics Resources (http://david.abcc.ncifcrf.gov/content.jsp?file=citation.htm) to analyze 28 major cellular pathways expected to be present in macrophages.[40,41]

RESULTS

Macrophage Isolation and Characterization. Homogeneous populations of viable macrophages were isolated from Balb/c mice. Macrophage purity was determined to be greater that 95% of isolated cells through a comparison of FITC-labeled anti-mouse F4/80 macrophage specific antibodies to identify macrophages in comparison to total cells measured using DAPI labeling of double-stranded DNA (Figure S1 of the Supporting Information). Viability was measured by comparing fluorescence signals associated with cell esterase activity visible upon cleavage of calcein AM in comparison to FITC-labeled anti-mouse F4/80 macrophage specific antibodies and represents >90% of isolated macrophages following correction for signals associated with uncomplexed FITC-labeled antibody visible even in the absence of added cells.

In Vivo Functional Measurements of Macrophage Recruitment. To assess age-dependent alterations in the inflammatory response of macrophages, the extent of in vivo macrophage recruitment was measured following injection of an irritant (i.e., sterile Bio-Gel P-100 polyacrylamide beads) into the peritoneal cavity of Balb/c mice. The large size of the Bio-Gel P-100 polyacrylamide beads (45−90 μm) prevents possible artifacts associated with phagocytosis and results in the isolation of a population of macrophages that remain largely quiescent in comparison with other elicitation protocols using, for example, thioglycollate (TG).[42,43] The number of macrophages isolated from young (3−4 months) mice was compared with the number isolated from older animals near the average mouse lifespan (14−15 months).[44] Prior to elicitation, we isolated an average of $(1.3 \pm 0.2) \times 10^6$ and $(0.90 \pm 0.02) \times 10^6$ resident macrophages from each young and old mouse, respectively (Figure 1A). The observation that young mice yield more resident macrophages than aged mice is consistent with earlier observations.[13] Following elicitation, there are substantial increases in the number of isolated macrophages, equaling an average of $(9 \pm 2) \times 10^6$ and $(12.6 \pm 0.8) \times 10^6$

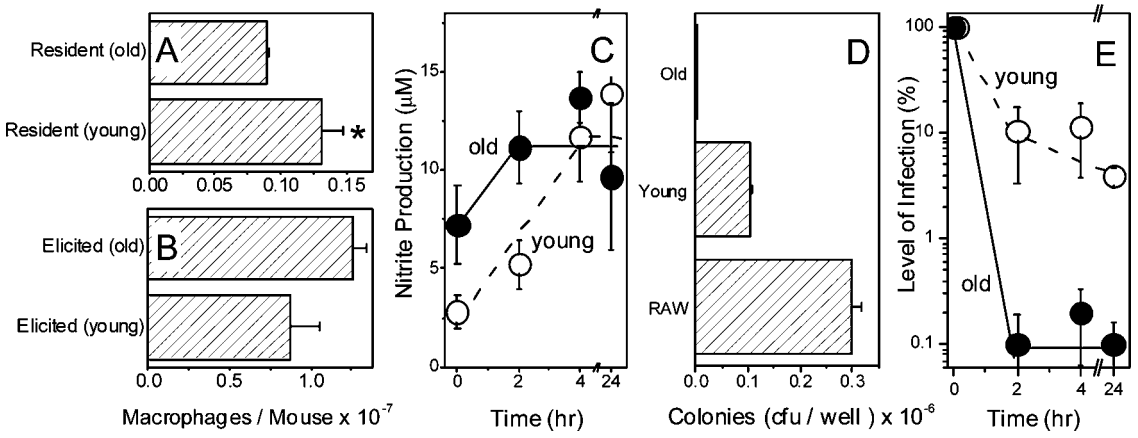

Figure 1. Yields and age-dependent functional changes for peritoneal macrophages. Average yields and variance of isolated peritoneal resident macrophages from young (3−4 months) and aged (14−15 months) Balb/c mice before (A) and following (B) challenge with Bio-Gel polyacrylamide beads (elicited response) for three independent experiments involving more than six mice in each experiment, where the asterisk indicates statistically significant differences based on a Student's t test ($p = 0.05$). (C) Nitric oxide production determined by measuring the release of the stable nitrite end product using Griess reagent and monitoring absorbance changes at 560 nm based on standard curves calibrated using sodium nitrite as a standard, as previously described.[22] (D) Surviving bacterial colonies measured 4 h after exposure and internalization of *S. typhimurium* 14028 cells for lysates prepared from 500000 isolated peritoneal macrophages or the RAW 264.7 macrophage cell line ($n = 9$), where the numbers of *S. typhimurium* colonies apparent for lysates prepared from 500000 macrophages following the 30 min incubation associated with infection were $(0.9 \pm 0.6) \times 10^6$ (young), $(1.4 \pm 0.5) \times 10^6$ (old), and $(2.4 \pm 0.1) \times 10^6$ (RAW 264.7). (E) Time dependence of surviving bacterial colonies measured following lysis of peritoneal macrophages ($n = 9$) infected with *S. typhimurium* 14028 cells. Peritoneal macrophages were isolated from young (3−4 months) ($n = 15$) and aged (14−15 months) BALB/c mice ($n = 11$).

Table 1. Tandem MS Identification of Peptides and Proteins[a]

	young		old		
MS/MS identification	NT	with LPS	NT	with LPS	all data sets
total no. of identified peptides	3133	3992	5601	6329	19055
no. of uniquely identified peptides	2062	2379	3781	4296	6578
no. of uniquely identified proteins	901	1096	1334	1459	2006[b]
no. of uniquely identified proteins	110	136	257	282	361[c]
linked to immune responses[c]	(12.2%)	(12.4%)	(19.3%)	(19.3%)	

[a]Number of total and uniquely identified peptides and corresponding proteins from tandem mass spectra from 30 SCX fractions from tryptic digests of lysates from peritoneal macrophages isolated from young or old mice prior to (NT) and following LPS activation, obtained using the 2006 international protein index (IPI) protein database (http://www.ebi.ac.uk.IPI/), as described in Experimental Procedures. [b]Includes 159 hypothetical proteins not included in the list summarizing 1847 known proteins that are found to be expressed in macrophages (Table S6 of the Supporting Information). [c]A complete listing of proteins identified as part of macrophage specific pathways is documented in Table S5 of the Supporting Information and involved searching a complete listing of identified proteins using the following search terms involving cytoskeleton/motility (search terms: transendothelia migration, cell motion, cell migration, and chemotaxis), phagocytosis/signaling (search terms: phagocytosis, ruffle, phagocytic cup, cell projection, and filopodium), antigen presentation/differentiation (search terms: antigen processing and presentation, antigen presentation, cytokine production, macrophage differentiation, complement, classical pathway, alternative pathway, and immunological synapse), and activation/stress response (search terms: immune cell activation, stress response, immune response, MAPKK, activation, macrophage, mast cell, platelet, B-cell, T-cell, tumor necrosis factor, interleukin, acute inflammatory response, and immunologic).

macrophages isolated from each young and old mouse, respectively (Figure 1B); overall yields are not statistically different, which is in agreement with prior reports that indicate age-dependent differences in the yield of resident macrophages disappear following elicitation.[13] These results indicate an age-dependent enhancement in the recruitment of macrophages into the peritoneal cavity of aged animals (14 ± 1-fold) in comparison to the young control (7 ± 2-fold), yielding equivalent numbers of macrophages. Age-dependent increases in the extent of macrophage recruitment may be relevant to understanding previous measurements that have identified age-dependent increases in cellular inflammatory responses and an enhanced resistance of infected mice to introduced bacterial pathogens.[10]

Ex Vivo ROS Generation and Bactericidal Activity. To assess possible age-dependent alterations in macrophage function, we measured the level of nitric oxide production, measured as total nitrite (Figure 1C). We observe increased levels of nitric oxide production during the first 2 h following exposure to bacterial endotoxin LPS. These latter results are in agreement with suggestions that increases in the level of generation of reactive oxygen species by macrophages contribute to age-dependent increases in the level of oxidative damage to cellular proteins.[45] At longer times, comparable levels of nitric oxide are detected irrespective of the age of the donor animals. These observations indicate that the age-dependent increase in the level of nitric oxide production does not represent an intrinsic limitation in the capacity of macrophages isolated from the younger animal to produce nitric oxide. Rather, macrophages isolated from aged animals are highly responsive to activation upon exposure to the bacterial endotoxin LPS.

Additional clarification of functional differences involved measurements of bacterial killing following their phagocytosis, which involves increases in the level of nitric oxide production as part of the oxidative burst. These measurements involved assays of the bactericidal response of isolated macrophages following phagocytosis of live bacteria (i.e., *S. typhimurium*). Macrophages were plated at the same density in all cases (i.e., 500000 macrophages/well). At various times following phagocytosis, macrophages were lysed and colonies that arose from surviving intracellular bacteria were measured using a plate assay, as previously described.[25] In comparison to a commonly used macrophage-like cell line derived from Abelson leukemic virus-induced tumors in Balb/c mice (i.e., RAW 264.7 cells), all isolated peritoneal macrophages have a substantially increased bactericidal activity that is indicative of highly coordinated metabolic pathways that are retained in aged animals (Figure 1D). However, macrophages isolated from old mice effectively kill 99.9% of phagocytosed bacteria during the first 2 h following infection (Figure 1E). In comparison, macrophages isolated from young control animals kill only 90% of the phagocytosed bacteria. An increased level of bacterial killing is consistent with observations that there is an enhanced basal level of nitric oxide production and a more rapid response following exposure to LPS. These results, in total, suggest that measurements of protein abundance changes following LPS exposure provide a realistic indication of age-dependent changes in macrophage response pathways.

Protein Identification and Pathway Interrogation. To access mechanisms associated with increased levels of recruitment and pathogen killing in macrophages isolated from aged mice, we have identified expressed proteins using tandem mass spectrometry. To achieve in-depth proteome coverage and to build an AMT database, we fractionated proteolytically digested macrophage lysates using strong anion exchange (SCX) chromatography, permitting the facile identification of 19055 peptides using a 1% false-discovery threshold ($q < 0.01$) (Table 1; see Figure S2A of the Supporting Information).[27,32] The 19055 identified peptides correspond to 6578 unique peptides that were used to build an accurate mass and elution time (AMT) database useful for quantitative measurements of protein abundance changes (see below), which match the masses of individual peptide sequences and normalized elution times to permit unique peptide identifications.[33] Identified proteins demonstrate, as expected, that pathways involving macrophage function are substantially enriched (Table S2 of the Supporting Information).

Using gene ontology software to link individual proteins to cellular function, we have identified 1847 macrophage proteins (see Table S6 of the Supporting Information). Irrespective of aging or macrophage activation, the distribution of proteins within gene ontology categories is very similar (Figure 2A,B). Nevertheless, there are substantial gaps in the proteome coverage, as we identify only 36% of the proteins in highly conserved central metabolic pathways (see Figure S3 of the

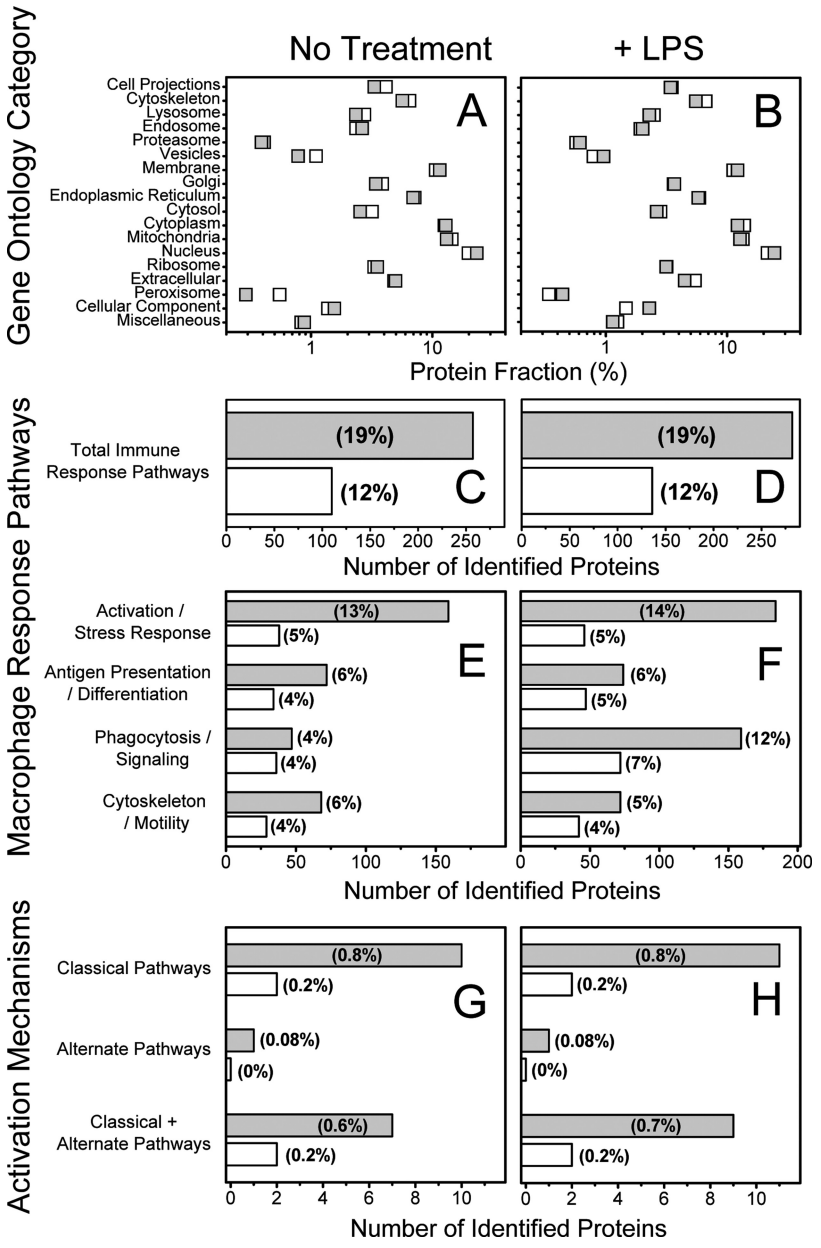

Figure 2. Age-dependent increases in the frequency of protein identifications for immune response pathways. Fractional contributions for protein classes within different gene ontology categories (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) for all 1847 identified proteins with annotated functions (see Table S6 of the Supporting Information) in peritoneal macrophages isolated from young (3−4 months) (n = 15) (open) and old (14−15 months) (n = 11) (gray) BALB/c mice following cell lysis and mass spectrometric protein identification for macrophages under resting conditions (no treatment) (A) or 2 h following exposure to LPS (B). (C−H) Number of proteins and fractional contributions (in brackets) for macrophage response pathways under resting conditions (C, E, and G) or following LPS activation (D, F, and H). Groupings were binned using gene ontology terms linked to all (total) immune response pathways (C and D) or specific terms relating to either the progressive activation of macrophage specific pathways (E and F) or the classical or alternative pathways of macrophage activation (G and H) (summarized in Table S5 of the Supporting Information). Proteins were counted more than once if annotations map onto multiple macrophage response pathways (E and F). Specific response pathways were identified by searching individual protein annotations using the following terms: cytoskeleton/motility (search terms: transendothelia migration, cell motion, cell migration, and chemotaxis), phagocytosis/signaling (search terms: phagocytosis, ruffle, phagocytic cup, cell projection, and filopodium), antigen presentation/differentiation (search terms: antigen processing and presentation, antigen presentation, cytokine production, macrophage differentiation, complement, classical pathway, alternative pathway, and immunological synapse), and activation/stress response (search terms: immune cell activation, stress response, immune response, MAPKK, activation, macrophage, mast cell, platelet, B-cell, T-cell, tumor necrosis factor, interleukin, acute inflammatory response, and immunologic).

Supporting Information). These results indicate that MS/MS proteomic measurements selectively identify the most abundant proteins, and that increases in the frequency of identification of proteins from specific pathways are indicative of increases in the number of proteins within these pathways. As a result, the substantial (>2-fold) increase in the number of identified proteins linked to immune response pathways for macrophages isolated from aged Balb/c mice, in comparison to young controls, suggests that there is an age-dependent upregulation of inflammatory pathways in macrophages that is broadly

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consistent with observed age-dependent increases in the level of inflammatory damage reported for a range of animal models (Figure 2C,D).

This is apparent from a consideration of identified proteins linked to antigen presentation as part of the MHC-1 pathway (see Figure S4 of the Supporting Information), where substantially more of the proteins linked to the formation of the immunoproteasome necessary for peptide degradation and antigen presentation are detected in macrophages isolated from aged animals (see Table S3 of the Supporting Information). Further, there are substantial increases in the number of detected proteasomal subunits following LPS exposure for macrophages isolated from aged mice; in comparison, LPS exposure has essentially no effect with respect to the number of identified proteosomal proteins in macrophages isolated from young animals. As antigen presentation is highly sensitive to macrophage activation,[46] these latter results are consistent with enhanced levels of macrophage activation in aged animals. Likewise, there are age-dependent increases in the number of identified proteins associated with immune response pathways within a range of macrophage response pathways, including those linked to cytoskeleton/motility, phagocytosis/signaling, antigen presentation/differentiation, and activation/stress response (Figure 2E,F). Within the list of identified proteins, there are also substantial age-dependent increases in the number of proteins assigned to play a role in the classical activation pathway (Figure 2G,H). However, for the majority of detected proteins, LPS exposure results in only modest (24–28%) increases in the fraction of identified proteins linked to these immune response pathways that is very similar for macrophages isolated from young or aged mice, suggesting the involvement of a subset of pathways (e.g., proteasome function) in age-dependent alterations in macrophage function. A similar insensitivity to LPS activation is apparent from a consideration of a range of proteins linked to antigen presentation pathways not involving the proteasome, which appear to be constitutively present irrespective of activation (Table S4 of the Supporting Information). These latter results are consistent with known regulatory control mechanisms in which pathway control typically involves the first committed step of a pathway (in this case antigen presentation as part of the MHC-1 immune response).

Identification of Protein Abundance Changes. Age-dependent differences in the abundance of specific proteins identified in both young and aged animals were resolved using quantitative proteomics using an accurate mass and time tag (AMT) database to compare ion currents of individual peptides.[33] In all reported proteins, abundance differences represent averages and standard deviations for more than two unique peptides in each protein, as previously described. For example, in the case of arginase 1, abundance changes during aging involve pairwise comparisons of ion current for seven different peptides, as fully described in Experimental Procedures (see Quantitative Measurements of Protein Abundance Changes) (Figure 3). Observed decreases in the peptide abundances for the seven resolved peptides vary from a 99.5% decrease (i.e., REGLYITEEIYK) to a maximal decrease in abundance of >99.9% (i.e., LKETEYDVRDHGDL). Collectively, the mean decrease in arginase I abundance is 99.7 ± 0.1%.

Following LPS exposure, there are significant changes in the abundance of 54 proteins, where 90% of affected proteins experience increases in their abundance in macrophages isolated from aged mice (Figure 4; see Table S1 of the Supporting Information). Of these proteins, we observe substantial

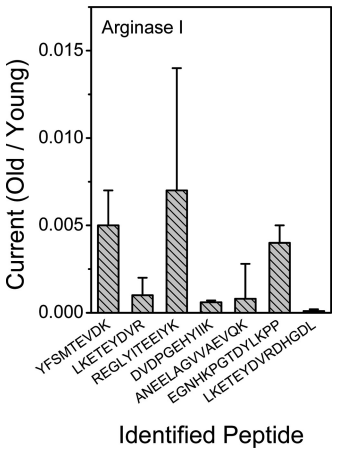

Figure 3. Age-dependent decrease in the abundance of arginase I. Abundance changes of seven individual peptides in arginase from lysates obtained from macrophages isolated from young and aged Balb/c mice (no LPS treatment) were obtained from a consideration of the ratio of their ion currents using the AMT reference database,[36] where averages and standard deviations are obtained for four technical replicate measurements, as fully described in Experimental Procedures.

increases in the abundance of diagnostic proteins linked to nitric oxide generation (e.g., annexin I, aldehyde dehydrogenase 2, and cystatin B) as well as coregulated antioxidant proteins that are part of the classical activation pathway of macrophage activation.[47−51] Further, of the 54 sensitive proteins, 26 have previously been colocalized within intracellular vacuoles associated with the compartmentalization and killing of bacterial pathogens.[52] The other 28 proteins that undergo age-dependent changes in abundance are all linked to known pathways associated with the macrophage immune response involving cytoskeletal motility, phagocytosis and signaling, antigen presentation and macrophage differentiation, and activation pathways involving reactive oxygen species and stress responses (see Table S1 of the Supporting Information). Thus, age-dependent alterations in macrophage function involving enhanced mobility, increases in the level of formation of nitric oxide, and higher rates of bacterial killing are all the result of a coordinated upregulation of normal pathways involving immune cell activation. There is no indication of any dysregulation of normal cellular pathways linked to macrophage activation.

Prior to macrophage activation by bacterial endotoxin LPS, a smaller number of proteins undergo changes in their abundance in macrophages isolated from aged mice, involving the downregulation of 11 proteins and the upregulation of 12 proteins (Figure 4). Of particular interest is the large decrease in the abundance of arginase I in macrophages isolated from aged animals (Figure 3), whose expression is under the control of cytosolic interleukins (such as IL-4) that act to induce a phenotypic switch that favors an alternative activation pathway.[3] This latter result is consistent with earlier observations that have demonstrated age-dependent declines in the ability of T-helper (CD4) cells to mount antigen specific Th2 responses involving the release of IL-4 and other cytokines that promote this alternative pathway of macrophage activation.[10] Rather, T-helper cells in aged animals primarily exhibit a Th1 phenotype involving the release of inflammatory cytokines IFNγ, IL-2, and TNFα that promote classical pathways of macrophage activation that enhance generalized antimicrobial responses involving the upregulation of iNOS. These latter results are

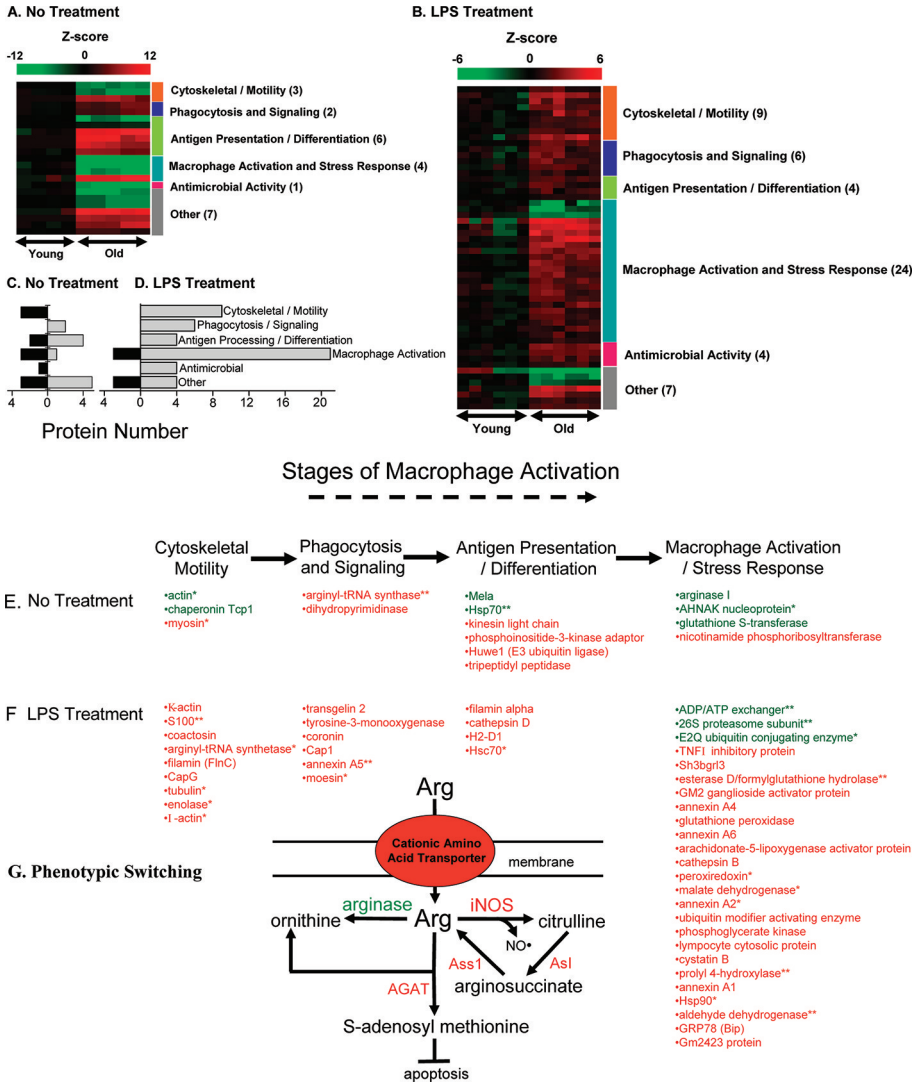

Figure 4. Selective age-dependent increases in the levels of macrophage proteins linked to antimicrobial responses. Heat map depictions of protein abundance changes (rows) for resting macrophages (No Treatment) (A) and 2 h following LPS exposure (LPS Treatment) (B) for macrophages isolated from aged (14−15 months) Balb/c mice (right panels) in comparison to macrophages isolated from young (3−4 months) Balb/c mice (left), comparing five or six independent mass spectrometric measurements of protein abundance changes (columns). The color code shown above each heat map represents a logarithmic (base 2) *Z* scale commonly used due to the ease of identification of 2-fold changes in abundance (i.e., $Z = 1$). The dynamic range of the *Z* scale varies between 0.0002 (i.e., $Z = -12$) and 4096 (i.e., $Z = 12$). Relationships between identified proteins that undergo age-dependent abundance changes to pathways linked to macrophage activation and antimicrobial responses are shown to the right of each heat map (A and B) and are summarized (C and D) to indicate the total numbers of proteins in each pathway that are upregulated (gray) or downregulated (black) in resting macrophages (C) or following LPS exposure (D). Proteins in heat maps are listed according to the magnitude of the abundance change within each identified pathway and are in the same order as in Table S1 of the Supporting Information, which indicate average abundance changes and describe protein functions. Age-dependent protein abundance changes were mapped onto antimicrobial response pathways, depicting decreases (green) or increases (red) in protein abundances for resting macrophages (E, no LPS treatment) or 2 h following exposure to bacterial endotoxin (F, LPS treatment) according to different cellular pathways known to be sequentially upregulated as part of the antimicrobial response. Indicated antimicrobial pathways involve (from left to right) abundance changes of cytoskeletal proteins linked to motility, increases in phagocytosis and signaling linked to bacterial sequestration, antigen presentation for sustained activation of T-helper cells, and final activation involving cytokine production, oxidative stress responses, and apoptosis. Proteins previously identified in *Salmonella*-containing vesicles (SCV) linked to bacterial killing are denoted with one or two asterisks, where two asterisks indicate that proteins were observed only following infection.[52] (G) Cartoon summarizing the age-induced phenotypic shift from alternative activation pathways (high arginase activity) to classical activation pathways (high nitric oxide levels). The cartoon is consistent with tandem MS identification of key enzymes involving arginine uptake, utilization, or recycling detected only in macrophages isolated from aged animals, including the cationic amino acid transporter associated with arginine uptake (entry 11987), argininosuccinate lyase (As1; entry 109900), arginine-glycine amidinotransferase (AGAT; entry 11987), and arginosuccinate synthetase (Ass1; entry 11898) (only following LPS activation). Enhanced *S*-adenosylmethionine levels resulting from AGAT activity protect macrophages against oxidative stress to decrease rates of apoptosis. Quantitative AMT data identify a 99% decrease in arginase (entries 11846 and 11847) abundance. Ornithine is metabolized to citrulline by ornithine carbamoyltransferase (within the urea cycle), not detected by tandem MS.

consistent with the functional data demonstrating higher levels of nitric oxide production in the resting state of aged macrophages as well as a hypersensitivity of macrophages isolated form aged animals to activation by LPS (Figure 1C).

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DISCUSSION

We report an age-dependent upregulation of coordinated pathways normally associated with macrophage activation, which provides a strong indication that increases in macrophage sensitivity to activation are fundamental to observed age-dependent changes involving innate immunity. These results do not support models involving genetically programmed cellular changes during aging that might be causal in the induction of a pathological state that involves a loss of coordination between normal pathways. Rather, our results support prior suggestions that "many of the aberrant responses seem to be dependent on the host environment, the milieu in which the cells reside, and might not be entirely dependent on the innate immune cells themselves".[53] Consistent with this model, our data suggest that environmental effects associated with immune cell exposure act to sensitize the macrophage to induce a chronic inflammatory response that is consistent with the vast majority of data concerning age-dependent phenomena. Apparent contradictory results in the literature can be understood in terms of differences in environmental exposure.

The data presented here represent the first global assessment of protein abundance changes of the aging process in macrophages, demonstrating an upregulation of immune pathways in macrophages isolated from aged mice. Prior to the identification of age-dependent changes in the macrophage proteome, we first confirmed prior observations of in vivo and ex vivo functional differences between peritoneal macrophages isolated from young (3−4 months) and aged (14−15 months) Balb/c mice (Figure 1). Observed age-dependent functional changes involving increases in (i) the extent of macrophage recruitment following elicitation, (ii) basal rates of nitric oxide generation that result in a reduction in the time needed for full activation of senescent macrophages following LPS exposure, and (iii) bactericidal activity following phagocytosis of *Salmonella* are all in agreement with prior observations, as well as suggestions that age-dependent increases in the levels of generation of reactive oxygen species by macrophages contribute to age-dependent increases in the levels of oxidative damage to a range of biomolecules.[9,10,13,45] Using these characteristic macrophage samples, it is therefore possible to employ proteomic measurements that identify specific proteins and related pathways to investigate fundamental mechanisms responsible for age-dependent changes in macrophage function.

To quantitatively address possible changes in immune specific pathways, protein abundance changes were measured using the quantitative AMT tag approach. We observe age-dependent abundance changes in 77 proteins known to play central roles in promoting macrophage activation (Figure 4; Table S1 of the Supporting Information). Virtually all identified proteins that undergo age-dependent abundance changes map onto normal processes associated with macrophage activation in terms of processes (Figure 4) or cellular locations (i.e., 26 of the 54 proteins whose abundances are altered during aging following LPS exposure colocalize with intracellular vacuoles associated with bacterial killing, a process linked to macrophage activation).[52] As reflected in the heat maps in Figure 4, observed abundance differences are highly reproducible (Figure 3; see the errors listed in Table S1 of the Supporting Information). Of particular interest is the large 99% decrease in the abundance of arginase I in macrophages isolated from aged animals, expression of which is under the control of cytosolic interleukins (such as IL-4) that act to induce a phenotypic switch that favors an alternative activation (Th2-like) pathway. These latter results are consistent with substantial increases in the abundance of diagnostic proteins linked to nitric oxide generation (e.g., annexin I, aldehyde dehydrogenase 2, and cystatin B) as well as coregulated antioxidant proteins that are part of the classical (Th1-like) macrophage activation pathway. The vast majority of the other proteins that undergo age-dependent changes in abundance are linked to known pathways associated with the macrophage immune response involving cytoskeletal motility, phagocytosis and signaling, antigen presentation and macrophage differentiation, and activation pathways involving reactive oxygen species and stress responses (Figure 4) that are consistent with observed age-dependent changes in macrophage function (Figure 1).

Specific examples of the mechanistic relationship between the quantitative AMT data and macrophage function include the identification of increases in the abundance of central proteins linked to normal pathways involving macrophage activation (see Table S1 of the Supporting Information). Consistent with age-dependent increases in macrophage elicitation and trafficking, we observe abundance increases in major structural proteins necessary for cytoskeletal motility that include actin, specific myosin isoforms linked to motility, tubulin, and macrophage capping protein (known to be critical for resisting infection). Likewise, age-dependent increases in bactericidal activity are consistent with quantitative increases in the abundance of key proteins that have previously been colocalized within intracellular vacuoles associated with bacterial killing.[52] These proteins include moesin (linked to TNF production), coronin (actin binding protein linked to phagosome formation), and transgelin (actin binding protein that suppresses MMP-9 and whose expression is triggered by TNF). Key proteins indicative of the upregulation of activation pathways associated with antigen presentation and macrophage polarization resulting from classical activation pathways include filamin α (initiates actin polymerization to reorganize cytoskeletal complexes that promotes MAPK-dependent signaling and ERK phosphorylation), cathepsin D (endopeptidase in lysosomes that promotes apoptosis through activation of caspase 8), histocompatibility-2 (surface glycoprotein linked to antigen processing and presentation via MHC-1 immune response), and heat shock protein 8 (involved in protein transport to the endoplasmic reticulum critical for antigen presentation). Observed age-dependent increases in the levels of nitric oxide generation are consistent with abundance changes for proteins linked to the respiratory burst and adaptive macrophage functions involving chaperones and antioxidants that are critical to macrophage activation, which include arginase I (decreases in abundance are diagnostic of a classical activation pathway, as arginase depletes arginine to inhibit nitric oxide production by NOS), cathepsin B (increases in the level of cysteine protease linked to antigen degradation and inflammatory processes associated with trafficking TNF-containing vesicles to the membrane), arachidonate-5-lipoxygenase activating protein (downstream of Toll receptors this protein plays a key role in forming leukotrienes linked to inflammatory responses), annexin A2 (promotes tyrosine kinase activation), lymphocyte cytosolic protein (actin binding protein critical to adhesion-dependent respiratory burst), and aldehyde dehydrogenase (antioxidant protein linked to nitric oxide production). Collectively, these results indicate a coordinated upregulation of normal pathways involving macrophage activation and do not support models that emphasize a

dysregulation of normal cellular pathways linked to macrophage activation.[3]

Observed age-dependent alterations in macrophage function are consistent with known age-dependent changes in the adaptive immune system, which involve shifts in T-helper cellular responses that favor the production of the inflammatory cytokines IFNγ, IL-2, and TNFα (i.e., Th1 response).[10] Such soluble factors induce the activation of resting macrophages to enhance nitric oxide production, antigen presentation, enhanced cytokine biosynthesis, and phagocytosis.[54] Thus, age-dependent increases in the resistance of macrophages to pathogens following their phagocytosis (Figure 1D,E),[9] as well as the increased resistance of old Balb/c mice themselves to introduced infections,[10] are consistent with well-documented age-dependent changes involving population shifts in T-lymphocytes to favor Th1 inflammatory responses. Apparent contradictory results in the literature, which detect both increases and decreases in macrophage function,[9,14,15,55−57] can be understood in terms of environmental factors and assay conditions that uncover age-dependent differences in macrophage sensitization to activating signals (e.g., LPS). For example, increases in nitric oxide levels, routinely observed upon challenge of elicited peritoneal macrophages by bacterial endotoxin[14] (Figure 1C), are abolished if macrophages are first uniformly sensitized by IFNγ exposure prior to endotoxin challenge.[8] Likewise, increases in the bacterial resistance of aged mice are dependent on environmental exposures to normal pathogens and are lost when mice are housed under sterile (clean room) conditions.[10] These observations are consistent with the considerable phenotypic heterogeneity present within macrophage populations, which is modulated in response to environmental conditions, which include responses to the generation of inflammatory cytokines (e.g., INF-γ, IL-2, and TNFα) and other soluble factors from T-lymphocytes that act to induce changes in the macrophage proteome and differentiation.[58,59]

What is currently unclear in the etiology of immune dysregulation during aging is the role that macrophages may play in promoting long-term shifts in T-lymphocyte population heterogeneity. Understanding the causal relationships that lead to age-related changes in the immune system requires an understanding of the functional coupling between the innate and adaptive immune systems. Specifically, antigen presenting cells (i.e., macrophages, dendritic cells, and B-cells) inform and amplify adaptive immune system responses and have the potential to induce shifts in innate and adaptive immunity characteristic of those seen in aged animals. For example, maintenance of macrophages in their basal state and their rapid activation in response to pathogen detection are central to the innate immune system, acting to limit nonspecific oxidative damage and promote pathogen killing following infection. In aged animals, macrophages exhibit a hyper-responsive and coordinated initiation of classical macrophage activation pathways, which enhance nitric oxide production and bactericidal activity to minimize infection from general microbial threats,[9,10] which are likely to be exacerbated by age-dependent decreases in the efficacy of physical barriers that limit pathogen entry to enhance environmental exposures. Upon pathogen recognition, macrophages release cytokines (e.g., IL-12) to induce T-helper cell differentiation to favor a Th1 response and release of inflammatory cytokines by T-helper cells that is associated with macrophage activation and T-helper cell proliferation in response to pathogen entry (Figure 5). Cross-talk with T-helper cells thereby amplifies

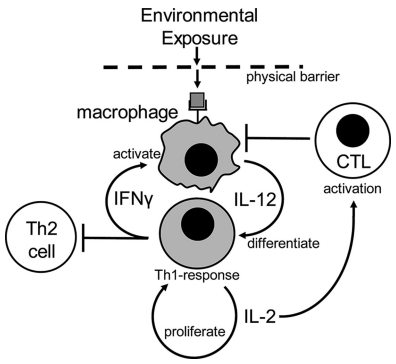

Figure 5. Aging induces persistent macrophage activation and reprogramming of adaptive cellular response. Age-dependent disruptions in physical barriers increase rates of pathogen entry, which upon recognition by macrophages (e.g., via Toll-like receptors) induce cytokine release (e.g., IL-12) to induce T-helper cell differentiation and Th1 responses involving the release of inflammatory cytokines (IFNγ and IL-2). IFNγ exposure enhances macrophage activation in the presence of bacterial antigens (e.g., LPS) and downregulates Th2 cellular responses by T-helper cells. The release of IL-2 by T-helper cells induces their proliferation favoring Th1 cellular responses and activation of cytotoxic T-cells (CTL), thereby amplifying the innate immune response and killing of infected (antigen presenting) macrophages.

inflammatory signals that promote macrophage activation in response to age-dependent decreases in physical barriers to pathogen entry that enhance exposure and further modify adaptive immunity through responses that originate in the innate system. These results suggest that therapies aimed to alleviate immune system dysregulation should include strategies aimed at neutralizing these amplification cascades between macrophages and T-lymphocytes, involving key targets of macrophage function that include not only inflammatory cytokines but also key signaling pathways involving macrophage activation, and pathways associated with antigen presentation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Documentation regarding macrophage purity (Figure S1), the statistical analysis used to identify macrophage proteins (Figure S2), proteomic coverage of 18 conserved pathways (Figure S3), identified proteins in antigen presentation pathways (Figure S4), functions and statistics for proteins that undergo age-dependent abundance changes (Table S1), documentation of enrichment for abundant pathways found in macrophages (Table S2), age-dependent differences in the identification of proteasome subunits (Table S3), tandem MS identification of central proteins in MHC-1 and MHC-II antigen presentation pathways (Table S4), proteins identified using tandem MS involved in immune-dependent pathways (Table S5), and an exhaustive listing of all 1847 proteins identified by tandem MS to be present in peritoneal macrophages isolated from Balb/c mice (Table S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

AMT, accurate mass and elution time; BRM, bioinformatics resource manager; cfu, colony forming unit; FBS, fetal bovine serum; FDR, false discovery rate; FITC, fluorescein isothiocyanide; IFNγ, interferon γ; IL, interleukin; iNOS, inducible nitric oxide synthase; LB, Luria broth; LC, liquid chromatography; LPS, lipopolysaccharide; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SCX, strong anion exchange chromatography; TCEP, tris(2-carboxyethyl)-phosphine; TG, thioglycollate; TNFα, tumor necrosis factor α.

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