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DAP12 Is Required for Macrophage Recruitment to the Lung in Response to Cigarette Smoke and Chemotaxis toward CCL2

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DAP12 is an adapter protein that associates with several receptors in macrophages. Little is known about the biological role of DAP12 in alveolar macrophages. In genome-wide profiling, we previously found that two DAP12-associated receptors, myeloid DAP12-associated lectin-1 and triggering receptor expressed on myeloid cells 2 (TREM2), were highly induced in alveolar macrophages from habitual smokers. Here, we found that transcript levels for these receptors in alveolar macrophages increased with packs per day of cigarettes smoked and expression of TREM2 protein was increased in lung macrophages of former smokers with emphysema compared with that in controls. In vitro, cigarette smoke directly induced expression of myeloid DAP12-associated lectin-1 and TREM2 and activation of DAP12 signaling in mouse macrophages. To determine whether DAP12 plays a role in cigarette smoke-induced pulmonary inflammation, we exposed wild-type and DAP12-deficient mice to chronic cigarette smoke and found significant reduction in recruitment of alveolar macrophages in DAP12-deficient mice. Because cigarette smoking induces the macrophage chemoattractant CCL2, we tested the chemotactic ability of DAP12-deficient macrophages and found abrogation of chemotaxis toward CCL2 in vitro. Airway administration of CCL2 also resulted in a significant reduction of macrophage recruitment to the lungs of DAP12-deficient mice compared with that in controls. DAP12 was also required for normal macrophage migration in a "scratch" assay. Reconstitution studies revealed that phosphorylation of the DAP12 ITAM was required for normal migration in vitro and association with TREM2 was sufficient for normal migration. These findings indicate that DAP12, possibly through association with TREM2, contributes to alveolar macrophage chemotaxis and recruitment to the lung and may mediate macrophage accumulation in lung diseases such as emphysema. *The Journal of Immunology*, 2010, 184: 6522–6528.

T he adapter protein, DAP12, contains an ITAM in its cytoplasmic domain and is highly expressed in NK cells and myeloid cells (reviewed in Refs. 1–5). DAP12 has a minimal extracellular domain and pairs with many different receptors through noncovalent interactions of the transmembrane domain of DAP12 and its associated receptors. In macrophages, there are at least eight known receptors that associate with DAP12 (reviewed in Ref. 3); however, ligands for many of these receptors remain unknown, a fact that has slowed progress in understanding the biological functions of DAP12 in macrophages. Initiation of DAP12 signaling in macrophages is thought to occur by cognate ligand binding to DAP12-associated receptors leading to phosphorylation of the tyrosines in the DAP12 ITAM by Src family kinases. The phosphorylated ITAM provides docking sites for the SH2 domains of spleen tyrosine kinase (SYK), which in turn leads to downstream signal transduction (reviewed in Refs. 6, 7). The biochemical signaling molecules responsible for the pro- or anti-inflammatory effector functions attributed to signaling through different DAP12-associated receptors are largely unknown (8–11). However, these data suggest that DAP12 may participate in significant cross-talk between other macrophage signaling pathways to regulate activation or inhibition of downstream pathways.

Despite our general understanding of DAP12 signaling, the functional consequences mediated by DAP12 in alveolar macrophages are unknown. We became interested in the role of DAP12 signaling in alveolar macrophages based on our prior work, which revealed that two DAP12-associated receptors were markedly induced in alveolar macrophages from habitual smokers, some of whom had chronic obstructive pulmonary disease (COPD) (12). COPD is a chronic lung disease characterized by airway inflammation leading to airway obstruction and emphysema. Because cigarette smoking is a major risk factor for the development of COPD and alveolar macrophages are required for cigarette smoke-induced COPD in mice, we explored how DAP12 contributed to macrophage inflammation in a mouse model of cigarette smoke exposure. We found that DAP12 was required for normal recruitment of alveolar macrophages to the lung in response to cigarette smoke. We hypothesized this was due to abnormal migration by DAP12-deficient macrophages to the lung. Our studies have revealed that DAP12 is required for normal chemotaxis of macrophages toward CCL2 in vitro and in vivo and further that migration

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Abbreviations used in this paper: BAL, bronchoalveolar lavage; BMM, bone marrow-derived macrophage; C, control media; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke-conditioned media; FAK, focal adhesion kinase; GPCR, G protein-coupled receptor; HA, hemagglutinin; MDL-1, myeloid DAP12-associated lectin-1; NS, nonsmokers; Ptx, pertussis toxin-treated; PY, anti-phosphotyrosine Ab; S, smokers; SHS, secondhand cigarette smoke; SYK, spleen tyrosine kinase; TREM2, triggering receptor expressed on myeloid cells; WT, wild type.

is dependent upon DAP12 phosphorylation. We also found that association of DAP12 with the DAP12-associated receptor triggering receptor expressed on myeloid cells 2 (TREM2) was sufficient to restore normal migration in vitro. These data reveal a novel regulator of macrophage migration and suggest biochemical crosstalk between DAP12 and CCR2 signaling.

Materials and Methods

Human alveolar macrophage isolation for quantitative real-time PCR

Human alveolar macrophages for quantitative real-time PCR analyses were obtained by bronchoalveolar lavage (BAL) in 30 research volunteers (15 smokers and 15 nonsmokers) as described previously (12).

Human lung tissue sections

Sections of paraffin-embedded lung tissue were obtained from the Lung Tissue Research Consortium (eight healthy controls and 27 subjects with COPD [diagnosis confirmed by spirometry; COPD severity was as follows: $n = 7$ mild, 7 moderate, 7 moderate/severe, and 6 severe]). Immunohistochemical localization of TREM2 was performed using rabbit anti-TREM2 Ab (Sigma-Aldrich, St. Louis, MO). After tissue sections were deparaffinized and rehydrated, they were immersed in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) and microwaved on high for 4 min, four times. Tissue sections were blocked with 5% human serum and 3% BSA in PBS for 30 min at room temperature and incubated for 1 h at room temperature with primary Ab and then for 1 h with biotinylated goat anti-rabbit secondary Ab (Vector Laboratories) followed by avidin–biotin complex reagent (Vector Laboratories). The sections were then incubated for 9 min in diaminobenzidine reagent (Zymed Laboratories, South San Francisco, CA) and counterstained for 5 s with Gill's no. 3 hematoxylin (Fisher Scientific, Hampton, NH). Quantification of immunostaining was performed independently with a ×40 objective (×400 total magnification) by two blinded investigators (P.G.W. and L.H.) on a scale of 0–4 based on the intensity of staining in macrophages in the sections (0 denoting absence of staining and 4 denoting maximally intense staining). All of the human studies were performed with approval of the University of California at San Francisco Committee on Human Research.

Mice and in vivo models

Wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). DAP12-deficient mice ($Tyrobp^{-/-}$) (13) were backcrossed to C57BL/6 for nine generations. Intranasal CCL2 was administered at 2 μg per dose for a total of four doses over 2 wk. Eight-week-old littermates were used for all of the experiments. BAL and total cell and differential counts were performed as previously described (14). The cigarette smoke exposure was conducted at the Institute of Toxicology and Environmental Health inhalation facilities (University of California at Davis, Davis, CA) in accordance with institutional guidelines as previously described (15). In brief, we used a smoke exposure system that provided for whole-body exposure (16). Eight-week-old littermate mice balanced between groups for gender were exposed to filtered air or cigarette smoke for 5 d/wk, 6 h/d. BAL cell counts collected from the wild-type mice used as concurrent controls in our smoke exposure experiment have been described previously (15). All of the mouse studies were performed with approval of the University of California at San Francisco Institutional Animal Care and Use Committee.

Cell culture

The mouse macrophage cell line RAW264.7 (American Type Culture Collection, Manassas, VA) was stably transfected with a hemagglutinin (HA) epitope-tagged mouse DAP12 cDNA construct (10) by using Amaxa's nucleofection protocol (Amaxa, Gaithersburg, MD). RAW DAP12-HA cells were cultured in DMEM with 4 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and 10% heat-inactivated characterized FBS. Puromycin was used in the growth medium at a concentration of 10 μg/ml to select for stable transfectants. Bone marrow-derived macrophages (BMMs) were generated from 6- to 8-wk-old wild-type and $Tyrobp^{-/-}$ C57BL/6 mice as previously described (11). Cells were grown on tissue culture-treated dishes and re-fed with DMEM media containing 10% heat-inactivated characterized FBS, 10% CMG (12–14 cell supernatant), L-glutamine, sodium pyruvate, penicillin, and streptomycin after 5 d, and macrophages were used in experiments from days 8–10. No differences in cell viability by trypan blue exclusion or annexin V staining were found between wild-type and DAP12-deficient macrophages.

Immunoprecipitation and Western blot

RAW DAP12-HA cells were grown to confluency on 15-cm cell culture-treated dishes. To stimulate macrophages in vitro with components of cigarette smoke, we generated media containing constituents of cigarette smoke as follows: 2R4F research cigarettes were mechanically "puffed" (following Federal Trade Commission conditions) in specially designed exposure chambers at the University of California at Davis as described previously (15). Cigarette smoke was pumped through filters to collect the smoke constituents. The filter was preweighed, and the net weight was recorded. The filters were then incubated in RPMI 1640 media for 1 h at 37˚C, and the concentration of smoke constituents was determined based on the net filter weight divided by the total volume of media. Cigarette smoke media did not contain LPS as determined by *Limulus* amebocyte lysate assay (detection limit of ~0.003 ng). Cells were stimulated with control or 250 μg/ml cigarette smoke media for 1 h. Cells were then lysed with RIPA lysis buffer containing 2 mM sodium orthovanadate and Protease Inhibitor Cocktail Set V (Calbiochem, La Jolla, CA). Lysate protein concentrations were determined (DC Protein Assay; Bio-Rad, Hercules, CA). Normalized lysates were precleared with protein G-Sepharose (GE Healthcare, Piscataway, NJ), and then immunoprecipitation was done with 5 μg anti-HA Ab (InvivoGen, San Diego, CA) and protein G-Sepharose for 16 h at 4˚C. The samples were then washed with PBS, and the pelleted protein G-Sepharose was resuspended in 2× Laemmli sample buffer, heated, and then separated by 4–20% SDS-PAGE and transferred to nitrocellulose. Blots were initially probed with biotinylated anti-phosphotyrosine Ab clone 4G10 (Millipore, Billerica, MA) and were then stripped using Restore Plus Western Blot Stripping Buffer (Pierce, Rockford, IL) and reprobed with goat anti-DAP12 Ab (sc-7855; Santa Cruz Biotechnology, Santa Cruz, CA) as a loading control or anti-SYK Ab (sc-1077, Santa Cruz Biotechnology), using appropriate secondary Abs for detection.

Flow cytometry

Wild-type RAW264.7 cells were exposed to either control media or 50 μg/ml cigarette smoke media for 24 h at 37˚C. Cells were analyzed by flow cytometry using biotinylated isotype-matched controls or biotinylated goat anti-mouse myeloid DAP12-associated lectin-1 (MDL-1) (BAF1639; R&D Systems, Minneapolis, MN) and biotinylated sheep anti-mouse TREM2 (BAF1729; R&D Systems) with PE-conjugated streptavidin (eBioscience, San Diego, CA) as a secondary Ab. Prior to Ab incubation, cells were incubated with mouse FcR blocking reagent (anti-CD16+CD32 mAb 2.4G2) as per the manufacturer's instructions (Miltenyi Biotec, Auburn, CA).

Adhesion assay

BMMs were plated at 40,000 cells per well on 96-well plates coated with fibronectin (BD Biosciences, San Jose, CA) in phenol red-free, serum-free DMEM with L-glutamine, sodium pyruvate, penicillin, and streptomycin. Cells were incubated at 37˚C for 30 min, and then macrophages were stained with Calcein AM (BD Biosciences) in PBS at a final concentration of 1 μM. Macrophages were again incubated at 37˚C for an additional 15 min. The first fluorescence measurement was then performed on a microplate reader with a 485/530 nm excitation/emission filter. The plate was then centrifuged, topside down, at 50 relative centrifugal force for 5 min. One hundred microliters of phenol red-free, serum-free DMEM was then added to the wells, and fluorescence was measured. The difference in fluorescence was determined, and the percentage of macrophages remaining adherent was calculated.

Migration assay

A total of 200,000 BMMs were added to the top-side of a 24-well, 8-μm pore size tissue culture-treated Transwell Permeable Support (Costar, Corning, NY) in DMEM with 1% BSA. DMEM with 1% BSA, with or without CCL2 at 100 ng/ml (R&D Systems), was added to the bottom chamber. Plates were incubated for 1.5 h at 37˚C. After incubation, the top sides of the Transwell membranes were thoroughly scraped with a micropipette tip, and the bottom surfaces were fixed and stained with Diff-Quick Stain Set according to the manufacturer's recommendations (Dade Behring, Newark, DE). Transwell membranes were mounted, and macrophages were counted in a blinded manner. Experimental groups were performed in triplicate, and counts represent an average, across the three replicates, of 10 random fields counted at ×400 magnification. The experiment was repeated three independent times, and the data from all of the experiments were averaged together.

Scratch assay

Macrophages were analyzed as previously described (17). In brief, BMMs were incubated in media with 100 ng/ml pertussis toxin (BIOMOL, Plymouth Meeting, PA) or vehicle control at 37˚C for 1 h. Then, macrophages

were plated in a 24-well tissue culture-treated plate in complete growth medium to attain ~80% confluency. Cells were allowed to adhere for 2 h and then were scraped with a P-1000 micropipette tip. Nonadherent cells were aspirated away, and complete growth media was replaced. Macrophages were then incubated at 37°C, and serial images were taken at designated time points at ×100 magnification. With Image J software (freeware; National Institutes of Health, Bethesda, MD), a standardized grid was placed over the images across the breadth of the denuded zone, and cells migrating into this zone were counted in a blinded fashion. Each quantified sample represents counts from four different wells with two serial images taken per well.

Retroviral transduction assay

Retroviral supernatants were generated by transient cotransfection of 293T cells with the pMIG-W vector containing flag epitope-tagged wild-type or different forms of mutant DAP12 and plasmids containing Moloney murine leukemia virus gag-pol and vesicular stomatitis virus G envelope with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Mutant DAP12 constructs included tyrosine to phenylalanine mutations in the DAP12 ITAM domain (Y65,76F) (18), an aspartic acid to alanine mutation in the transmembrane domain of DAP12 (D52A) (10), and a DAP12 chimera consisting of the extracellular portion of TREM2 fused to the DAP12 transmembrane mutant (10). For macrophage reconstitution, bone marrow was collected from wild-type and DAP12-deficient mice. Cells were cultured for 24 h in DMEM with 10% FCS containing penicillin, streptomycin, and CMG supernatant. Suspended cells were incubated for 12 h at 37°C in 5% CO_2 with retroviral supernatants supplemented with 8 g/ml of polybrene and medium conditioned with monocyte CSF. The viral supernatant was replaced with culture medium, and cells were differentiated for 8–10 d. Infected macrophages were analyzed by flow cytometry for GFP expression and expression of flag epitope-tagged DAP12 showing >60% transduction efficiency for each experiment. Infected macrophages were detached from plates with cell-dissociation buffer (Invitrogen) and used in scratch assays.

Statistical analysis

We used the Student t test (two-tailed) for pairwise comparison of group means. For three or more groups, we first applied one-way ANOVA and then applied the t test, corrected for multiple testing (Sidak correction), for pairwise comparisons. To test for a stepwise increase across three groups, we performed the nonparametric trend test. Analyses were performed using STATA 9.0 (StataCorp, College Station, TX), and $p < 0.05$ was considered statistically significant.

CCR2 flow cytometry

Eight- to 10-d-old BMMs and PBMCs from 2-mo-old wild-type and DAP12-deficient mice were incubated with mouse FcR blocking reagent as per the manufacturer's recommendations (Miltenyi Biotec) followed by incubation with CCR2 PE mAb (clone 48607) or recommended isotype control (clone 133303) (R&D Systems) as per the manufacturer's protocol. Flow cytometry was performed using a FACScalibur, and data were analyzed using FlowJo, version 7.2.4.

Peripheral blood monocyte counts

Whole blood was obtained from 8-wk-old mice by cardiac puncture and analyzed for monocyte levels on the Hemavet Multispecies Hematology Analyzer 850FS (CDC Technologies–Drew Scientific, Centerville, OH).

Immunofluorescence

One million BMMs were plated on a 22-mm circular cover glass resting in wells of a six-well plate with complete growth media and allowed to adhere at 37°C for 24 h. A line of confluent cells was scraped off with a P-1000 micropipette tip, nonadherent cells were removed, and the "wounds" were allowed to heal for 24 h at 37°C. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton-X. For actin visualization, cells were blocked with a 2% BSA solution, and then actin was stained with rhodamine phalloidin (Molecular Probes, Eugene, OR). For paxillin and focal adhesion kinase (FAK) staining, cells were blocked with a mouse FcR blocking reagent according to the manufacturer's protocol (Miltenyi Biotec). Cells were stained with rabbit anti-human FAK polyclonal Ab (BioSource International, Carlsbad, CA), followed by biotinylated anti-rabbit IgG (Vector Laboratories) and then with streptavidin-conjugated Oregon Green 488 (Molecular Probes) or anti-human paxillin mouse mAb (clone Z035; Zymed Laboratories), followed by Alexa Fluor 592 anti-mouse (Molecular Probes) to visualize FAK and paxillin, respectively. After being stained, cover slips were mounted with Vectashield hard set mounting medium with DAPI (Vector Laboratories). All of the images were acquired at the Nikon Imaging Center at the University of California at San Francisco.

Results

Expression of DAP12-associated receptors are increased by chronic cigarette smoking and COPD

We previously identified upregulation of mRNA encoding two DAP12-associated receptors in genome-wide expression analysis of human alveolar macrophages from habitual cigarette smokers (12). Out of >54,000 probe sets that we analyzed, *CLEC5A* (MDL-1) and *TREM2* were among the most highly induced genes in smokers' macrophages with transcript levels increased >3-fold compared with those of nonsmokers (12). Quantitative RT-PCR confirmed a significant increase in normalized gene copy number for *CLEC5A* (Fig. 1A) and *TREM2* (Fig. 1B) in these samples. In addition, transcript levels for *CLEC5A* and *TREM2* were directly proportional to packs of cigarettes smoked per day (Fig. 1A, 1B). The gene for
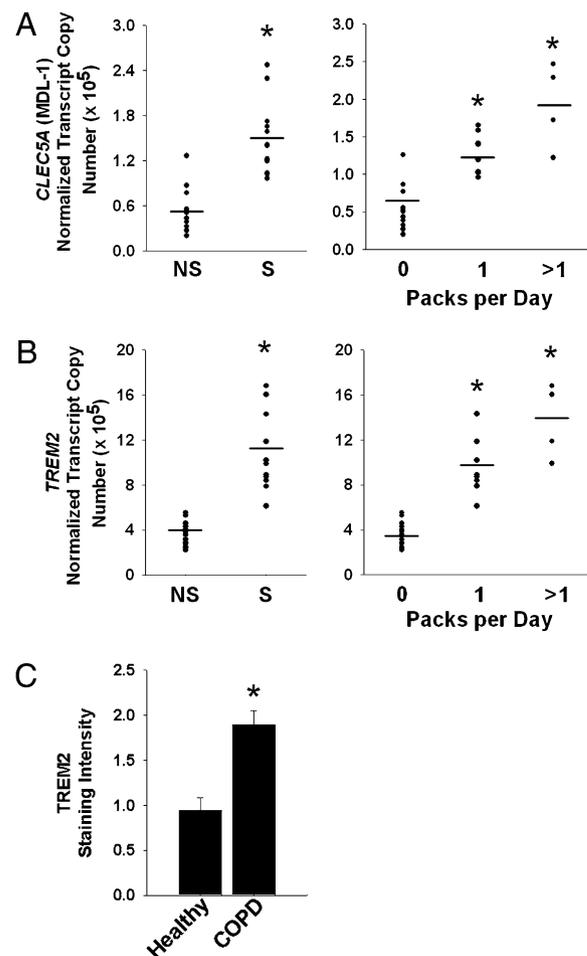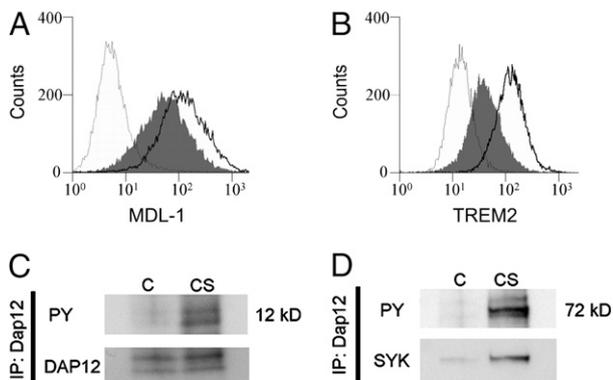

FIGURE 1. Habitual cigarette smoking leads to a dose-related increase in expression of DAP12-associated receptors in alveolar macrophages. *A* and *B*, Alveolar macrophages were isolated by bronchoscopy and purified by flow cytometry (12). Total RNA was isolated and mRNA transcript levels for *CLEC5A* (MDL-1) and *TREM2* were measured by quantitative RT-PCR. Transcript levels for both receptors were increased in smokers' macrophages compared with those of nonsmokers (A, B, *left panel*) and increased in proportion to the numbers of packs per day smoked (A, B, *right panel*). *$p <$ 0.0001; *$p <$ 0.001 using nonparametric trend test, which tests stepwise increase in each group. Data are expressed as normalized transcript copy number; bars represent means. $n = 15$ for NS and 12 for S. *C*, Lung tissue sections were stained for TREM2 protein using anti-TREM2 Ab or isotype-matched control Ig, and macrophage staining intensity was independently scored by two blinded investigators. Data are expressed as staining intensity $\pm$ SE. $n = 8$ healthy subjects and 27 former smokers with COPD. We did not find significant associations of staining intensity with COPD severity (as reported by the Lung Tissue Research Consortium). *$p <$ 0.005. NS, nonsmokers; S, smokers.

DAP12 (*TYROBP*) was detectable by quantitative RT-PCR but not induced, suggesting that signaling through DAP12 is regulated by the expression of receptors as opposed to DAP12 itself. In lung sections from former smokers with COPD and control nonsmokers without COPD, we found that macrophages lined the alveolar spaces and that the intensity of immunostaining for TREM2 protein was homogeneously increased in these macrophages in COPD as compared with that in controls (Fig. 1*C*, Supplemental Fig. 1). Attempts to detect human MDL-1 by immunohistochemistry using commercially available reagents were unsuccessful.

To test whether the induction of DAP12-associated receptors was due to direct effects of constituents of cigarette smoke on macrophages, we developed an in vitro model of cigarette smoke exposure. Exposing a mouse macrophage cell line (RAW264.7) to aqueous components of cigarette smoke, we found increased protein expression for MDL-1 and TREM2 at 24 h (Fig. 2*A*, 2*B*). Furthermore, we found that components of cigarette smoke led to activation of the DAP12 pathway as assessed by tyrosine phosphorylation of DAP12 and its downstream signaling mediator SYK (Fig. 2*C*, 2*D*). No other cell types were required for this effect, demonstrating that this is a cell autonomous response. Together, these in vitro data indicate that cigarette smoke exposure directly induced expression of MDL-1 and TREM2 in macrophages and activated the DAP12 pathway. Our human in vivo data indicate that cigarette smoke exposure increased alveolar macrophage MDL-1 and TREM2 expression in a dose-dependent manner. Further, TREM2, at the protein level, was induced in former smokers with COPD, suggesting that the DAP12 pathway may play a role in modulation of alveolar macrophage function even after smoking cessation.

DAP12 is required for recruitment of alveolar macrophages to the lung in response to cigarette smoke

The biological effects of DAP12 signaling in alveolar macrophages are unknown. To further explore the role of DAP12 in the de-
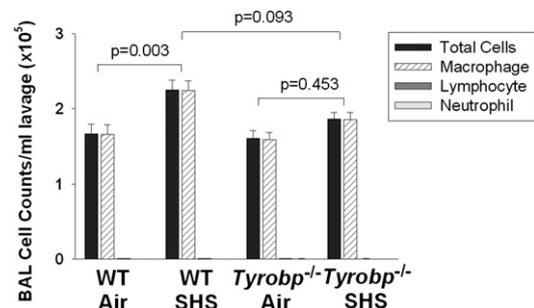
velopment of macrophage inflammation, we used a mouse model of chronic cigarette smoke exposure. Our primary end point was bronchoalveolar inflammation induced by cigarette smoke after 3 mo of exposure. In wild-type mice, exposure to cigarette smoke induced a ~30% increase in total bronchoalveolar inflammatory cells compared with that of the filtered air control (Fig. 3). However, recruitment of inflammatory cells to the lungs by cigarette smoke in DAP12-deficient mice was reduced by >50% compared with that in cigarette smoke-exposed wild-type littermates. Differential cell counts revealed that the majority of recruited cells were macrophages (Fig. 3). These data indicate that DAP12 is required for normal recruitment of alveolar macrophages to the lung in response to cigarette smoke.

We hypothesized that this defect was due to aberrant migration of DAP12-deficient macrophages to inflammatory mediators induced by cigarette smoke. In a previous study, we identified that cigarette smoke exposure in mice led to significant increases in BAL levels of CCL2 (15), a known chemoattractant for macrophages. Therefore, we tested whether DAP12 mediates chemotaxis. We found that DAP12-deficient macrophages were unable to chemotax across a Transwell in response to a gradient of CCL2, whereas wild-type macrophages showed a significant increase in chemotaxis (Fig. 4*A*). We found no difference in chemokinesis between wild-type and DAP12-deficient macrophages when CCL2 was placed in the upper and lower wells (data not shown). On the basis of these data, we measured alveolar macrophage recruitment in vivo in response to intranasal instillation of CCL2. Compared to wild-type mice exposed to intranasal CCL2, ~50% fewer macrophages were recruited into the lungs of DAP12-deficient mice treated with CCL2 (Fig. 4*B*). The magnitude of the recruitment defect in DAP12-deficient mice in response to CCL2 was similar to that measured in response to cigarette smoke (Fig. 3). In addition, we found no difference in the levels of CCR2 expression on PBMCs from wild-type and DAP12-deficient mice or wild-type and DAP12-deficient macrophages used in the chemotaxis assay (Supplemental Fig. 2). Furthermore, there were no significant differences in the levels of CCL2 in BAL fluid from cigarette smoke-exposed wild-type and DAP12-deficient mice (16.2 ± 16 versus 25.1 ± 23 pg/ml, respectively; $p = 0.2$). Taken together, these data reveal a novel role for DAP12 in the recruitment of alveolar macrophages in response to cigarette smoke that may be mediated through chemotaxis toward CCL2.

To further examine the migratory ability of DAP12-deficient macrophages, we performed scratch assays in which an area of macrophage monolayer is scratched off and the ability of cells to migrate into the scratched zone is quantified. This assay revealed a significant decrease in the ability of DAP12-deficient macrophages to migrate into the denuded zone compared with that of wild-type

FIGURE 2. Constituents of cigarette smoke increased MDL-1 and TREM2 surface expression on macrophages and phosphorylated DAP12 in vitro. RAW264.7 cells were exposed to either control media or 50 μg/ml cigarette smoke-conditioned media for 24 h. *A* and *B*, Cells were analyzed by flow cytometry for (*A*) MDL-1 and (*B*) TREM2 surface expression. Data are displayed as flow cytometric histogram plots with gray line representing isotype-matched control Ab, black filled histogram representing MDL-1 or TREM2 Ab, and black line representing smoke media plus MDL-1 or TREM2 Ab. Data are representative of three independent experiments. *C* and *D*, RAW264.7 cells expressing an HA epitope-tagged DAP12 were exposed to C media or 250 μg/ml CS-conditioned media for 60 min. Lysates were immunoprecipitated with anti-HA Ab, and immunoprecipitates were analyzed by SDS-PAGE. Membranes were blotted with PY, then stripped and reblotted with (*C*) anti-DAP12 Ab as a loading control or (*D*) anti-SYK Ab (showing increased coimmunoprecipitation of SYK with phospho-DAP12). Data are representative of three independent experiments. C, control; CS, cigarette smoke; PY, phosphotyrosine Ab.
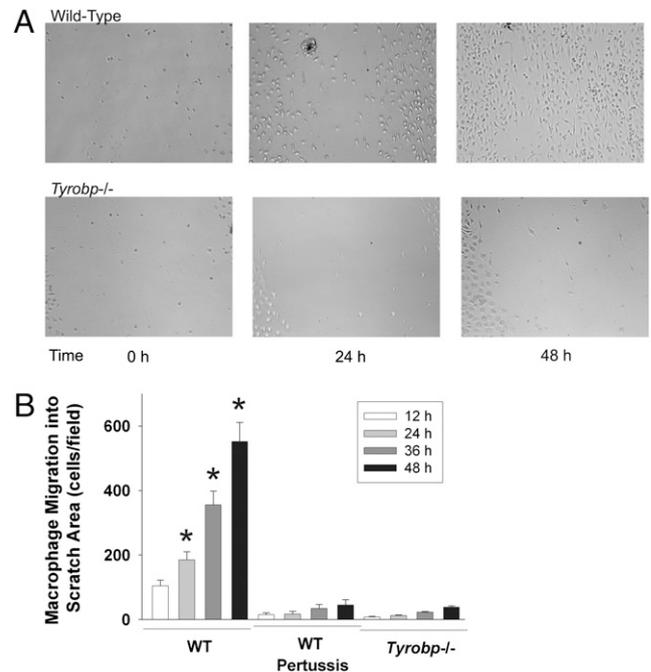

FIGURE 3. DAP12 is required for normal recruitment of macrophages to the lung in response to cigarette smoke. Wild-type and DAP12-deficient (*Tyrobp*$^{-/-}$) mice were exposed to 3 mo of SHS, and numbers of BAL cells were quantified ($n = 16$ per group). Data are expressed as cell counts per milliliter of lavage ± SE. SHS, secondhand cigarette smoke.
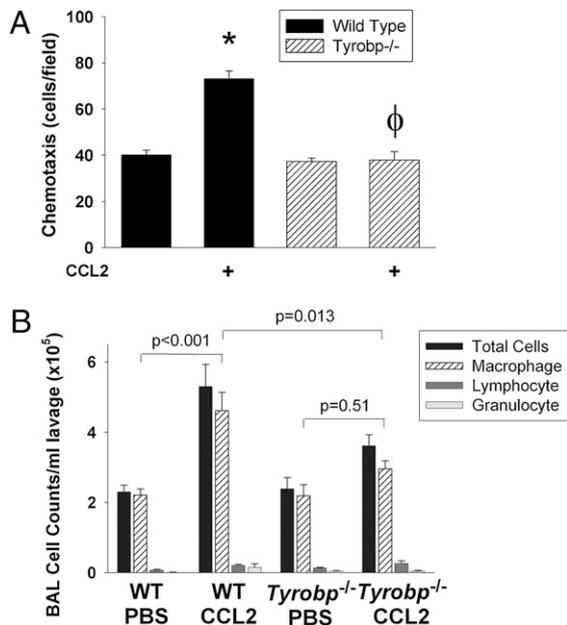
FIGURE 4. Abnormal chemotaxis and recruitment of DAP12-deficient macrophages to CCL2 in vitro and in vivo. *A*, Chemotaxis of wild-type and DAP12-deficient BMMs was assessed using Transwells containing culture medium with or without CCL2. Data are expressed as mean ± SE of nine replicates for each condition (three independent experiments, each done in triplicate). *$p < 0.001$ compared with wild-type and DAP12-deficient controls; $\phi p < 0.001$ compared with wild-type cells exposed to chemoattractants and $p > 0.8$ compared with DAP12-deficient control. *B*, Wild-type and DAP12-deficient mice ($Tyrobp^{-/-}$) were given CCL2 intranasally over 2 wk, and numbers of BAL cells were quantified ($n = 6$–7 per group). Data are expressed as cell counts per milliliter of lavage ± SE.


FIGURE 5. DAP12-deficient BMMs demonstrated significantly reduced migration in an in vitro scratch assay. *A* and *B*, Macrophages were cultured to confluence and incubated with or without pertussis toxin as described in *Materials and Methods*. Macrophage monolayers were "wounded" by scratching with the tip of a pipette. Serial images of cells were taken immediately (time 0) and at the indicated times after "wounding." Images of the scratch area are shown (*A*), and macrophage migration into the scratch area is quantified (*B*). Data in *B* are expressed as numbers of macrophages migrating into scratch area (defined as counts at given time point minus counts at time 0). Data are representative of three independent experiments. $p < 0.001$ compared with other conditions at same time point adjusted for multiple comparisons. Ptx, pertussis toxin-treated; WT, wild type.

macrophages (Fig. 5). To assess the relative contribution of G protein-coupled receptor (GPCR)-mediated chemotaxis observed in the scratch assay, wild-type macrophages were treated with pertussis toxin, and migration in the scratch assay was quantified. Fig. 5*B* shows a reduction in migration of pertussis toxin-treated-wild-type macrophages to a level observed with DAP12-deficient macrophages. No significant decrease in DAP12-deficient macrophage proliferation was found using BrdU labeling (percentage BrdU labeling, wild-type 78 ± 4% versus DAP12-deficient 93 ± 2%). Furthermore, treatment of DAP12-deficient macrophages with the GSK3b-specific inhibitor SB216763, which promotes M-CSF–mediated proliferation of macrophages, did not increase the number of migrating macrophages into the scratch area compared with that in vehicle control-treated cells ($p > 0.1$ at all time points; data not shown), indicating that defects in proliferation do not account for the decreased migration of DAP12-deficient macrophages.

To determine the requirements for DAP12 receptors and ITAM phosphorylation in mediating migration in the scratch assay, we performed a number of reconstitution experiments using primary macrophages. Wild-type and DAP12-deficient macrophages were transduced with retroviral vectors containing wild-type or mutant DAP12. Reconstitution of wild-type DAP12 into DAP12-deficient macrophages restored migration into the scratch area similar to that of wild-type macrophages transduced with the control vector (Fig. 6). Transduction of DAP12-deficient macrophages with a DAP12 ITAM tyrosine mutant (Y65,76F) that cannot be phosphorylated resulted in a lack of migration similar to that measured in control DAP12-deficient macrophages (Fig. 6). In addition, migration was also inhibited in DAP12-deficient macrophages transduced with a DAP12 transmembrane mutant that cannot associate with receptors (D52A) (10). Finally, reconstitution of DAP12-deficient

macrophages with a TREM2/DAP12 chimera containing the DAP12 transmembrane domain mutation (which allows functional DAP12 signaling to be restricted to TREM2) restored migration to a level similar to that observed in macrophages reconstituted with wild-type DAP12 (Fig. 6). Taken together, these data indicate that signaling through the ITAM of DAP12 as well as association with DAP12 receptors are required for migration into the scratched area and that association with TREM2 is sufficient for migration.

We performed further experiments to examine whether additional mechanisms might contribute to the reduced macrophage recruitment in DAP12-deficient mice. We found no differences in the number of circulating monocytes in DAP12-deficient mice compared with that in controls (Supplemental Fig. 3). We also found no differences in the ability of DAP12-deficient macrophages to adhere to integrin ligands in vitro, including fibronectin (wild-type versus DAP12-deficient mean percentage adherence ± SD, 78 ± 7% versus 79 ± 5%, respectively). Furthermore, DAP12-deficient macrophages had no obvious defects in formation of actin stress fibers or focal adhesions (Supplemental Fig. 4) and spread normally in culture, whether at high or low density (Supplemental Fig. 5).

Discussion

Although DAP12 was first described a decade ago (4), the biological functions mediated by DAP12 and its associated receptors in alveolar macrophages are unknown. In this report, we present evidence that several DAP12-associated receptors are induced in alveolar macrophages from habitual cigarette smokers in a dose-related fashion. In addition, we found that increased protein expression for TREM2 persists in former smokers with COPD. In vitro studies
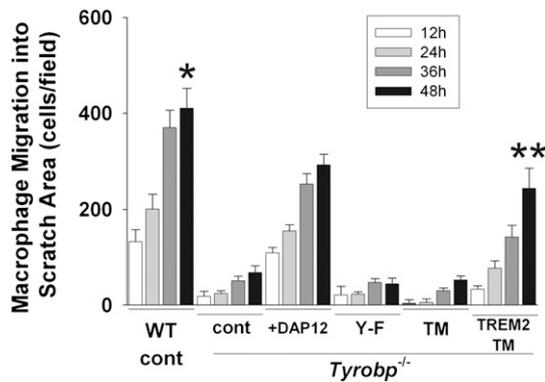
FIGURE 6. Reconstitution of DAP12-deficient macrophages with DAP12 mutants reveals a role for TREM2 and phosphorylation of DAP12 ITAM in migration in vitro. Wild-type and DAP12-deficient macrophages were transduced with retroviruses containing a vector with one of the following mutants: empty vector (cont), wild-type DAP12 (+DAP12), DAP12 Y65,76F tyrosine ITAM mutations (Y-F), DAP12 D52A transmembrane mutation (TM), and TREM2/DAP12 chimera containing a DAP12 transmembrane mutation (TREM2 TM). Migration of cells was quantified in the scratch assay. Data are expressed as numbers of macrophages migrating into scratch area (defined as counts at given time points minus counts at time 0). Transduction efficiency was determined by flow cytometry to be ∼60–70% for each condition. Data are representative of three independent experiments. $*p < 0.0001$ compared with $Tyrobp^{-/-}$ control, Y-F and TM and p value was not statistically significantly different compared with $Tyrobp^{-/-}$ +DAP12 at the same time point adjusted for multiple comparisons; $**p$ not statistically significant compared with $Tyrobp^{-/-}$ +DAP12.

established that the effect of cigarette smoke on the upregulation of DAP12-associated receptors and activation of the DAP12 pathway in macrophages was direct, requiring no other cell types. To understand how the DAP12 pathway may contribute to smoking-induced macrophage inflammation, we used a mouse model of chronic cigarette exposure known to induce CCL2 in the lung (15) and found that DAP12 regulates cigarette smoke-induced recruitment of alveolar macrophages. We also found that DAP12 was required for in vitro and in vivo chemotaxis of macrophages toward CCL2. Further, we identified that phosphorylation of the ITAM of DAP12 and association of DAP12 with receptors were necessary for migration in vitro. Overall, these data demonstrate the role of DAP12 in the recruitment of alveolar macrophages to the lung in response to cigarette smoke and CCL2 that might be mediated through association with TREM2 and possibly other DAP12-associated receptors.

Recruitment of macrophages to the lung in response to cigarette smoke is thought to be important in the development of COPD (19–27). We and others have previously found that cigarette smoke can induce genome-wide transcriptional changes in alveolar macrophages from chronic cigarette smokers, including the upregulation of genes encoding DAP12-associated receptors. In the current study, we have performed experiments that have identified DAP12 as an important mediator of pulmonary macrophage recruitment. Our data also identify a defect in chemotaxis toward CCL2, a known monocyte/macrophage chemoattractant (28–30). These findings suggest potential biochemical cross-talk between DAP12 and CCR2 signaling and are congruent with a prior study that showed that the ITAM adapter CD3ζ not only associated with the chemokine receptor, CXCR4, but was also required for CXCR4 signaling (31). Thus, ITAMs may be important in the modulation of GPCR signaling and downstream cellular functions such as chemotaxis. Future studies that examine interactions between DAP12 and other GPCRs will elucidate whether DAP12 plays a more general role in chemotaxis of macrophages to other chemokines.

The role of DAP12 in modulating specific cellular functions may, in part, be cell type-specific, dependent on different DAP12-associated receptors, or both. For example, we have shown previously that DAP12 plays a partial role in mediating adhesion of neutrophils to fibronectin (32) as well as chemokine-independent recruitment of neutrophils to the peritoneum (33). However, in macrophages, we found that DAP12 was not required for adhesion to fibronectin or formation of actin stress fibers or focal adhesions. Because DAP12 is known to associate with at least eight receptors in myeloid cells (3), these cell type-specific functions of DAP12 may reflect divergent roles of specific DAP12-associated receptors. For example, our data suggest that TREM2 is sufficient to mediate migration of macrophages. Thus, an important step in understanding the cell type-specific roles of DAP12 is to determine whether specific DAP12-associated receptors are required for each of these functions.

In addition to mediating chemotaxis, DAP12-associated receptors in alveolar macrophages may also be involved in the anti-inflammatory functions of macrophages within the lung. For example, we and others have previously found that TLR-induced production of TNF, IL-6, and IL-12 p40 is enhanced in BMMs that lack DAP12 (11) and that association of DAP12 with TREM2 restored TLR-induced cytokine production to normal levels (9, 10). In this prior study, we also found evidence for a TREM2 ligand on the surface of macrophages (10). Overall, these data suggest that DAP12 can mediate inhibitory signals and that ligands for some of the DAP12-associated receptors may be induced on the macrophage cell surface to regulate DAP12 function. In this study, we found that cigarette smoke induced phosphorylation of DAP12 at both early (1 min) and late (60 min) time points, indicating proximal phosphorylation by cigarette smoke and later phosphorylation events. At this point, we can only speculate on the mechanism(s) for these phosphorylation events that include ligand induction on the surface of macrophages or multireceptor complex formation (e.g., with CCR2) to modulate macrophage function.

In contrast to the anti-inflammatory functions identified for TREM2-induced signaling, DAP12 also has been shown to mediate activating signals consistent with the well-described activating function of ITAMs. This function was highlighted in a recent study by Chen et al. (8) where they identified dengue virus as an activating ligand for MDL-1 (CLEC5A), leading to the production of proinflammatory cytokines and mediators that play a role in the development of dengue fever sepsis syndrome. The signaling mechanisms to explain these differing responses have yet to be identified; however, it has been postulated that activating versus inhibitory signaling mediated by DAP12 may be regulated by the avidity of ligand binding to DAP12-associated receptors (1, 6, 7, 34).

The pro- or anti-inflammatory consequences of induction of both MDL-1 and TREM2 in macrophages by cigarette smoke are uncertain because they may play divergent roles or be important in distinct phases of inflammation or its resolution. Given that MDL-1 ligation can lead to proinflammatory cytokine production (8), it is possible that cigarette smoke-induced upregulation of MDL-1 leads to enhanced production of inflammatory mediators that contribute to emphysema, such as MMP-12 (data not shown). However, there is experimental evidence that TREM2 participates in repair processes that follow injury. Specifically, mouse models of experimental allergic encephalitis have found that TREM2 expression by microglial cells is required for normal clearance of apoptotic neurons (35, 36). More recently, Seno et al. (37) reported that TREM2 expression by macrophages was necessary for normal wound healing of colonic mucosa. In this model, injured colonic mucosa from TREM2-deficient mice showed increased markers of classical macrophage activation and decreased expression of cytokines that were associated with normal wound healing. Thus,

TREM2-mediated signals in macrophages may play important roles in facilitating resolution of inflammation. Our data that TREM2 expression persists in alveolar macrophages from former smokers with COPD are consistent with such a role in repair. Furthermore, our in vitro reconstitution studies suggest that TREM2 may be upregulated to facilitate macrophage migration to the lung in response to injury (such as by cigarette smoke) and participate in repair processes given the experimental data published to date. Future studies in TREM2- and MDL-1–deficient mice will be necessary to understand whether these receptors mediate specific pro- or anti-inflammatory functions in response to cigarette smoke and other forms of lung injury.

In summary, alveolar macrophages are increasingly recognized as important contributors to the development of lung diseases. By performing a genome-wide expression analysis of alveolar macrophages from habitual smokers, some of whom had emphysema, we identified upregulation of two DAP12-associated receptors. We found that DAP12 contributes to the recruitment of macrophages to the lung possibly through regulation of chemotaxis toward CCL2 and association of DAP12 with its receptor TREM2. In addition to understanding the biology of macrophage migration to the lung, these results have implications in treating conditions that involve macrophage migration, including conditions such as lung cancer, where tumor-associated macrophages have been linked to tumor proliferation and facilitating metastases (25–27).

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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