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Fas-Activated Serine/Threonine Phosphoprotein Promotes Immune-Mediated Pulmonary Inflammation

Maria Simarro,^{*,†} Giorgio Giannattasio,^{*,†,‡} Miguel A. De la Fuente,^{§,¶} Charaf Benarafa,^{||} Kulandayan K. Subramanian,^{#,**} Rumey Ishizawar,^{*,†} Barbara Balestrieri,^{*,†} Emma M. Andersson,^{*,†} Hongbo R. Luo,^{#,**} Antonio Orduña,^{††} Joshua Boyce,^{*,†} and Paul Anderson^{*,†}

We generated Fas-activated serine threonine phosphoprotein (FAST)-deficient mice (FAST^{-/-}) to study the in vivo role of FAST in immune system function. In a model of house dust mite-induced allergic pulmonary inflammation, wild type mice develop a mixed cellular infiltrate composed of eosinophils, lymphocytes, and neutrophils. FAST^{-/-} mice develop airway inflammation that is distinguished by the near absence of neutrophils. Similarly, LPS-induced alveolar neutrophil recruitment is markedly reduced in FAST^{-/-} mice compared with wild type controls. This is accompanied by reduced concentrations of cytokines (TNF- α and IL-6 and -23) and chemoattractants (MIP-2 and keratinocyte chemoattractant) in bronchoalveolar lavage fluids. Because FAST^{-/-} neutrophils exhibit normal chemotaxis and survival, impaired neutrophil recruitment is likely to be due to reduced production of chemoattractants within the pulmonary parenchyma. Studies using bone marrow chimeras implicate lung resident hematopoietic cells (e.g., pulmonary dendritic cells and/or alveolar macrophages) in this process. In conclusion, our results introduce FAST as a proinflammatory factor that modulates the function of lung resident hematopoietic cells to promote neutrophil recruitment and pulmonary inflammation. *The Journal of Immunology*, 2010, 184: 5325–5332.

Fas-activated serine/threonine phosphoprotein (FAST) is a mitochondria-associated protein that promotes the survival of cells exposed to adverse environmental conditions (1, 2). In stressed cells, FAST is released from mitochondria, allowing interactions with the translational repressor T cell intracellular Ag-1 (TIA-1). By inhibiting the activity of TIA-1, FAST promotes the translation of inhibitors of apoptosis. In this capacity, FAST acts as a survival protein that inhibits Fas- and UV-induced apoptosis (1, 2). In stressed cells, FAST and TIA-1 are colocalized in cytoplasmic stress granules, a compartment that helps to reprogram protein expression to promote survival under adverse environmental con-

ditions. Thus, FAST and TIA-1 are functional antagonists that help to determine whether stressed cells live or die.

FAST and TIA-1 are also found in the nucleus where they regulate the alternative splicing of exons flanked by weak splice site-recognition sequences. FAST promotes the inclusion of exon IIIb of the fibroblast growth factor receptor 2 mRNA and exon 6 of Fas mRNA (3, 4). Fas transcripts that include exon 6 encode a transmembrane receptor that promotes apoptosis. Fas transcripts lacking exon 6 encode a soluble receptor that inhibits apoptosis. By influencing levels of the soluble and membrane-associated Fas, FAST is likely to modulate the survival of immune cells at sites of inflammation.

FAST is overexpressed in pancreatic tumors, where its targeted knockdown represses the expression of transcripts encoding proteins involved in cell proliferation, adhesion, and motility (5). FAST is also overexpressed in the cutaneous T cell lymphoma mycosis fungoides (6). In both cases, overexpression of FAST may contribute to the transformed phenotype by promoting cell survival. Although TIA-1 has been characterized as a translational repressor, it also indirectly regulates levels of mRNA expression (7). Indeed, depletion of TIA-1 and T cell intracellular Ag-1 related protein (TIAR; a closely related homolog) promotes the expression of transcripts encoding proteins involved in inflammation, cell growth, and proliferation (8–10). Consequently, knockdown of TIA-1/TIAR in HeLa cells leads to increased proliferation, altered cell cycle times, and anchorage-independent growth (11). Thus, overexpression of FAST or reduced expression of TIA-1/TIAR leads to increased cell survival and proliferation.

FAST mRNA is also overexpressed in PBMCs derived from patients with asthma, atopy, type I diabetes, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (12, 13). It is possible that FAST contributes to these immune-mediated inflammatory diseases by promoting the survival of inflammatory cells and/or preventing TIA-1-mediated repression of soluble mediators of inflammation. Mutant mice lacking TIA-1 have an

^{*}Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, [†]Department of Medicine, and [‡]Department of Pediatrics, Harvard Medical School; [§]Division of Immunology, [¶]Department of Lab Medicine and Joint Program in Transfusion Medicine, and ^{**}Department of Pathology, Children's Hospital Boston, Boston, MA 02115; ^{‡‡}Department of Clinical Pathophysiology and Experimental Medicine, University of Naples, Federico II, Naples, Italy; ^{||}Theodor Kocher Institute, University of Bern, Bern, Switzerland; and ^{††}Unidad de Investigación, Hospital Clínico Universitario de Valladolid, Valladolid, Spain

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Address correspondence and reprint requests to Dr. Paul Anderson, Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Smith 652, One Jimmy Fund Way, Boston, MA 02115. E-mail address: panderson@rics.bwh.harvard.edu

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Abbreviations used in this paper: 7AAD, 7-aminoactinomycin D; ALI, acute lung injury; BAL, bronchoalveolar lavage; BM, bone marrow; Ctrl, control; DC, dendritic cell; E, Ecl 136II; EOS, eosinophils; ES, embryonic stem; FAST, Fas-activated serine threonine phosphoprotein; H, HindIII; HDM, house dust mite; HI, homing index; K, KpnI; KC, keratinocyte chemoattractant; LTB4, leukotriene B4; LYM, lymphocytes; MEF, mouse embryonic fibroblast; N, NotI; neo, neomycin resistance expression cassette; NEU, neutrophils; SI, SacI; TCN, total cell number; TIA-1, T cell intracellular Ag-1; TIAR, T cell intracellular Ag-1 related protein; TRITC, tetramethylrhodamine-5-(and-6)-isothiocyanate; WT, wild type; X, XhoI.

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inflammatory diathesis that is manifest by spontaneous arthritis (10). In this article, we show that mutant mice lacking FAST are resistant to neutrophilic pulmonary inflammation in a model of house dust mite (HDM)-induced allergic pulmonary inflammation and a model of LPS-induced neutrophil recruitment. These findings support the concept that FAST and TIA-1 are molecular antagonists that regulate cell survival and inflammation.

Materials and Methods

Generation of FAST^{-/-} mice

The targeting vector was designed to delete *Fastk*, replacing it with loxP-flanked neo expression cassette. To generate the 5' homology arm, a 3.3-kb fragment was amplified by PCR from the genomic DNA isolated from W4/129S6 embryonic stem (ES) cells (Taconic Farms, Germantown, NY) using the primers NotI 3353 (MSG 76) and SalI 6782 (MSG 77) (primer sequences are provided in Supplemental Table I). The PCR product was digested with NotI and SalI and cloned into NotI and SalI sites of pKO Scrambler. To generate the 3' homology arm, a HindIII/SacI fragment of 5.8 kb from a FASTK genomic clone isolated from a 129/SvJ genomic library (Stratagene, La Jolla, CA) was cloned into pBluescript II KS⁺ (Stratagene). This modified pBluescript II KS⁺ plasmid was digested with XhoI and Ecl136II to excise the 3' arm, which, in turn, was ligated into XhoI and HpaI sites of pKO Scrambler. pKO Scrambler vector (Stratagene) had been previously modified so that neo expression cassette was flanked by loxP sites. The targeting vector was linearized at a unique NotI site and electroporated into W4/129S6 ES cells (Taconic Farms), and cells were selected in medium containing 200 µg/ml G418 and 1 µM ganciclovir. Correctly targeted clones were identified by Southern blotting using a 5' external probe (227-bp PvuII/PvuII). Three targeted clones were identified among 192 colonies screened. Correctly targeted ES cell clones were injected into C57BL/6J(B6) blastocysts to generate chimeras. After germ-line transmission was confirmed by crosses with B6 females, the resulting progeny were bred with Cre-expressing mice (C57BL/6 EIIa-cre transgenic mice, stock #003724, The Jackson Laboratory, Bar Harbor, ME) to remove the neo cassette. The removal of neo in the resulting pups was confirmed by PCR using primers MSG418 and MSG419 (primer sequences are provided in Supplemental Table I). Sequencing of PCR products was performed to confirm the expected structural changes. FAST^{-/-} mice were backcrossed onto the B6 background (stock #000664, The Jackson Laboratory) for 12 generations.

Murine model of HDM-induced allergic pulmonary inflammation

Mice were sedated with 100 mg/kg ketamine and 10 mg/kg xylazine and challenged intranasally with 10 µg HDM extract (Greer Laboratories, Lenoir, NC) in 20 µl sterile saline for 2 d a week for three consecutive weeks. Twenty-four hours after the last challenge, mice were killed, and bronchoalveolar lavage (BAL) fluid, lungs, blood, and spleen were collected.

Murine model of LPS-induced lung injury

Sedated mice were challenged once with 200 µg/kg LPS from *Escherichia coli* 055:B5 (Sigma-Aldrich, St. Louis, MO) by intranasal instillation, and the mice were killed 20 h later for BAL and blood collection.

Bronchoalveolar lavage

Tracheas were cannulated with an 18-gauge angiocath. Lungs were lavaged three times with 0.7 ml cold PBS containing 0.5 mM EDTA. The cells were counted, cytopun, and stained with Diff-Quick (Dade Behring, Deerfield, IL) for differential analysis using morphologic criteria under a light microscope, with the evaluation of 500 cells/slide. For studies of cytokines in BAL fluid, the first wash was collected separately and centrifuged, and the supernatant was stored at -80°C until analysis.

Lung histology

The left lung lobes were removed, fixed in formalin, and processed for routine histology in paraffin. Sections were stained with naphthol AS-D chloroacetate esterase with counterstaining by hematoxylin to depict neutrophils.

Cell culture

RBC-depleted splenocytes (2×10^6 /ml) were incubated for 72 h in the presence of HDM extract (20 µg/ml). Supernatants were collected for ELISA analysis. Bone marrow (BM) neutrophils were purified from 8–12-

wk-old mice, as previously described (14), and cultured at 2×10^6 /ml for 24 h to study in vitro spontaneous apoptosis. Mouse embryonic fibroblasts (MEFs) were prepared from embryonic day 9.5 embryos.

Cytokine and Ig measurements

Cytokine levels in BAL fluid and splenocyte supernatants were measured by ELISA using matched Ab pairs from eBioscience (San Diego, CA; TNF-α, IL-6, and MIP-2) or R&D Systems (Minneapolis, MN; IL-23 and G-CSF) or by SearchLight Technology by Aushon (Billerica, MA; IL-1β, keratinocyte chemoattractant [KC], MCP-1, and IL-17A). Serum total IgE and HDM-specific IgG1 Ab titers were measured by ELISA, as previously described (15).

Flow cytometry

Blood leukocytes were stained with FITC-conjugated anti-Gr-1 (clone RB6-8C5) and PE-conjugated anti-Ly6G (clone 1A8). Gr-1 high Ly6G⁺ cells were neutrophils. Early apoptosis in BM neutrophils was quantified by the expression of membrane phosphatidylserine detected by Annexin V-FITC binding, as recommended by the manufacturer (BD Pharmingen, San Diego, CA). BM neutrophils were stained with PE-conjugated anti-Ly6G (clone 1A8). Because translocation of phosphatidylserine to the external cell surface also occurs during necrosis, it was used in conjunction with the 7-aminoactinomycin D (7AAD) nucleic dye, which stains nonviable cells. This allowed us to differentiate early apoptotic cells (Annexin V-FITC⁺, 7AAD⁻) from late apoptotic cells (Annexin V-FITC⁺, 7AAD⁺-bright). Stained cells were acquired on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and data were processed by FlowJo software (Tree Star, Ashland, OR).

Blood counts

EDTA-anticoagulated blood samples were used to obtain a complete blood count with a Hemavet Mascot Multispecies Hematology System Counter 1500R (CDC Technologies, Oxford, CT).

EZ-TAXIScan chemotaxis assay

Real-time horizontal chemotaxis assays were performed using the EZ-TAXIScan chamber (Effector Cell Institute, Tokyo, Japan). The EZ-TAXIScan chamber consists of an etched silicon substrate and a flat glass plate, both of which form two compartments with a 5-µm-deep microchannel. BM neutrophils (1 µl, 3×10^6 /ml) were put into one hole in the stainless steel holder with which the device is held together, and 1 µl chemoattractant (100 nM leukotriene B4 [LTB4] or 1 µM fMLP) was put into another contrahole. The chamber was incubated for 20 min at 37°C. To count the migrated cells in each channel, images of the cells in each channel were captured at 30-s intervals using the ×10 lens on a Discovery Screening System (Universal Imaging, Downingtown, PA). The x,y coordinates of migrating neutrophils were tracked from sequential images using DIAS imaging software (Solltech, Oakdale, IA). Average migration speed (µm/min; migration distance between the current frame and the previous frame divided by the time between sequential frames, 0.5 min) was calculated at each captured frame. Directionality was calculated by dividing the straight-line distance by the total curve-line distance that the cell moved. Upward directionality was calculated by dividing the straight-line distance that the cell moved in the upward direction divided by the total curve-line distance that the cell moved.

Neutrophil-homing assay

BM wild type (WT) neutrophils were labeled for 10 min at 37°C with 5 µM intracellular fluorescent dye CFSE (Molecular Probes, Eugene, OR), and BM FAST^{-/-} neutrophils were labeled for 10 min at 37°C with 7.5 µM tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC, Molecular Probes). Labeled WT (CFSE⁺) and FAST^{-/-} (TRITC⁺) cells were mixed at a 1:1 ratio, and 2×10^6 cells were injected i.v. into WT and FAST^{-/-} mice 6 h after being challenged with 200 µg/kg LPS intranasally. Two hours after injection, recipient mice were killed, BAL cell suspensions were analyzed by flow cytometry, and the percentages of CFSE⁺ and TRITC⁺ cells were determined. The homing index was calculated as the ratio of CFSE⁺/TRITC⁺ cells in the BAL fluid divided by the ratio of CFSE⁺/TRITC⁺ cells in the input.

Assay for production of reactive oxygen species

Production of reactive oxygen species was assayed by luminol-ECL. Mice 8–12 wk old were injected i.p. with 3% thioglycollate broth (2 ml) 4 h prior to peritoneal lavage with RPMI 1640. The proportion of neutrophils in the peritoneal lavage was >90%, judged by light microscopy of Diff-Quick-stained cytopspin preparations. The assay mixture (0.2 ml) contained

200 μ M luminol and 2×10^5 neutrophils in HBSS. Neutrophils were activated with zymosan (30 particles per cell; Sigma-Aldrich) in HBSS or HBSS as a control. Chemiluminescence was measured at the indicated intervals with a luminometer (Monolight 2010, Analytical Luminescence Laboratory, San Diego, CA) and expressed as relative luciferase unit per 1×10^4 cells.

Generation of chimeric mice

Three-week-old recipient mice were lethally irradiated in two doses of 600 rad each (separated by 4 h). BM from donor mice was harvested from femora and tibiae, and $\sim 6 \times 10^6$ cells in 0.2 ml were injected i.v. into recipient mice. BM transplantation was performed in four groups of mice: BM from FAST^{-/-} into WT (FAST^{-/-}→WT chimeric, expressing FAST on non-hematopoietic cells only); BM from WT into FAST^{-/-} mice (WT→FAST^{-/-} chimeric, expressing FAST on hematopoietic cells only); BM from FAST^{-/-} to FAST^{-/-} (FAST^{-/-}→FAST^{-/-}); and BM from WT to WT (WT→WT). Mice in the latter two groups served as negative and positive controls for possible radiation effects. To permit complete chimerism, we allowed 10 wk of reconstitution time before we started experiments (16). Peripheral blood counts and PCR analysis of blood DNA were performed to confirm the hematopoietic engraftment 4 wk after BM transplantation. We performed two separate PCR reactions to amplify a 384-bp fragment on the WT allele with the MSG128/MSG129 primer pair or a 669-bp fragment on the knockout allele with the MSG105/MSG133 primer pair (primer sequences are provided in Supplemental Table 1).

Northern blot, immunoblot, and real-time quantitative PCR analysis

FAST transcript and protein expression in MEFs were evaluated by Northern blot using a FAST cDNA fragment (nucleotides 1369–1621 of GenBank accession no. BC013547; www.ncbi.nlm.nih.gov/) and by immunoblot using goat polyclonal anti-FAST (Santa Cruz Biotechnology, Santa Cruz, CA). Relative mRNA expression levels of FAST in lung and neutrophils were measured by SYBR Green based real-time quantitative PCR assay. GAPDH, β -actin, β -2 microglobulin, hypoxanthine phosphoribosyltransferase 1, and 18S were used as housekeeping genes. The following primer pairs were used: MSG299/MSG300 (for FAST); MSG151/MSG152 (for GAPDH); MSG287/MSG288 (for β -actin); and MSG323/MSG324 (for hypoxanthine phosphoribosyltransferase 1) (primer sequences are provided in Supplemental Table 1). Primer pairs for β -2 microglobulin and 18S were purchased from Superarray Biosciences (Frederick, MD). Data were analyzed using GeNorm software (Primer-Design, Southampton, U.K.).

Statistics

All analyses were performed using Prism software (GraphPad, La Jolla, CA). Data are expressed as mean \pm SEM and were analyzed using one-

way ANOVA with Bonferroni correction or the unpaired Student *t* test, as appropriate.

Results

Generation of FAST^{-/-} mice

The genomic FAST locus contains 10 exons spanning 4.3 kb (Fig. 1A). We constructed a targeting vector designed to replace the entire FAST gene with a loxP-flanked neo expression cassette using flanking sequences isolated from W4/129S6 ES cell-derived genomic DNA. This vector was transfected into W4/129S6 ES cells for selection of stable integrants that were identified using Southern blotting (Fig. 1B) and PCR (Fig. 1C). Correctly targeted ES cell clones were injected into C57BL/6J(B6) blastocysts to generate chimeras. After germ-line transmission was confirmed by crosses with B6 females, the resulting progeny were bred with Cre-expressing mice (C57BL/6 Ella-cre transgenic mice) to remove the neo cassette. FAST^{-/-} mice were backcrossed onto the B6 background for 12 generations. MEFs prepared from FAST^{-/-} mice do not express FAST mRNA (Fig. 1D) or protein (Fig. 1E).

FAST^{-/-} mice are born at the expected Mendelian frequency and are morphologically indistinguishable from WT mice. Evaluation of peripheral blood cell counts revealed no significant differences between WT and FAST^{-/-} animals (data not shown). Similarly, cell surface marker analysis of cells recovered from BM, spleen, and thymus revealed no differences between FAST^{-/-} and WT mice in the numbers of cells committed to myeloid (Gr-1, and Mac-1) or lymphoid (CD45R/B220, CD3, CD4, and CD8) lineages (data not shown).

FAST promotes HDM-induced allergic pulmonary inflammation

The finding that FAST is overexpressed in PBMCs from patients with atopy, asthma, type I diabetes, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (12, 13) led us to examine the role of FAST in immune-mediated inflammatory disease. We chose the HDM Ag model of allergic pulmonary inflammation because it uses a clinically relevant allergen with endogenous adjuvants that break tolerance across the mucosal barrier (17, 18). Mice were exposed to intranasal HDM extract 2 d per week for 3 wk, a protocol that results in a Th2-associated

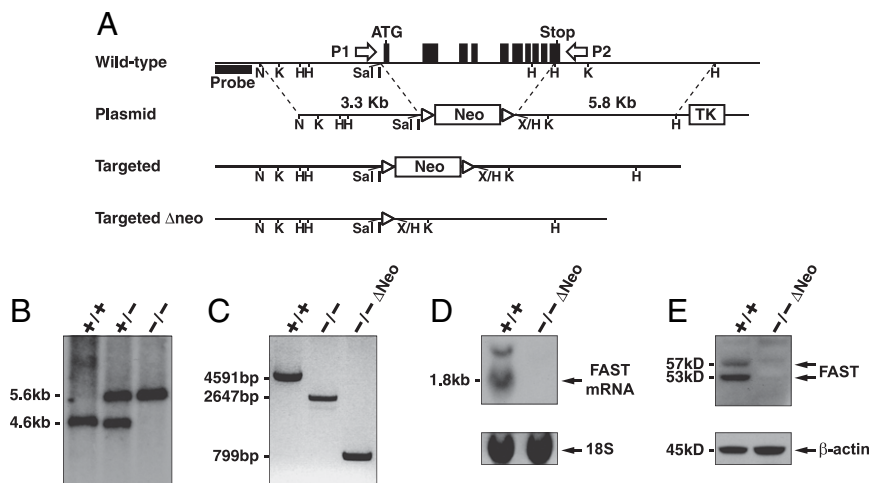


FIGURE 1. Generation of FAST^{-/-} mice. *A*, Gene-targeting strategy. Correct targeting will delete *Fast*, replacing it with a loxP-flanked neo cassette. The neo cassette is removed by crosses with Ella-Cre mice. Exons are indicated by black boxes. E, Ecl 136II; H, HindIII; K, KpnI; N, NotI; neo, neomycin resistance expression cassette; SI, SacI; X, XhoI; Δ , loxP site. *B*, Southern blot analysis of DraI-digested genomic DNA from WT (+/+), heterozygote (+/-), and FAST^{-/-} (-/-) mice. The probe used is shown in *A*. *C*, PCR analysis of tail DNA by using primers P1 (MSG418) and P2 (MSG419) distinguishes WT, FAST^{-/-} neo+ (-/-), and FAST^{-/-} neo- (-/- Δ neo) alleles. Primer sequences are provided in Supplemental Table 1. *D*, Northern analysis of mRNA from WT and FAST^{-/-} primary MEFs. *E*, Western blot analysis of protein extracts from WT and FAST^{-/-} MEFs with the indicated Abs.

airway inflammation characterized by BAL fluid and bronchovascular lymphocytosis, eosinophilia, neutrophilia, and goblet cell hyperplasia. In HDM-challenged WT mice, the infiltrates in BAL fluids consist of ~49% mononuclear cells, 45% eosinophils, and 6% neutrophils. Although the absolute number of inflammatory cells in BAL fluids obtained from WT and FAST^{-/-} mice was similar, compositional analysis revealed the virtual absence of neutrophils in FAST^{-/-} BAL fluid ($0.32 \times 10^4 \pm 0.19 \times 10^4$ cells versus $3.91 \times 10^4 \pm 0.35 \times 10^4$ cells; $p < 0.01$) (Fig. 2A). Histological analysis of the lungs confirmed the virtual absence of neutrophils in >95% of the bronchovascular bundles analyzed (Fig. 2B). In WT and FAST^{-/-} mice, HDM exposure increased total serum IgE and HDM-specific serum IgG1 to similar levels, indicating an intact immune response (Fig. 2C). Culture of WT and FAST^{-/-} splenocytes from HDM-challenged mice in the presence of HDM extract (20 μ g/ml) for 4 d resulted in the secretion of similar amounts of IL-4, -5, and -13 (Fig. 2D). Thus, FAST does not modulate the induction of Th2 responses and allergic eosinophilic airway inflammation to HDM, but it is required for neutrophil recruitment to inflamed lungs.

FAST promotes LPS-induced pulmonary inflammation

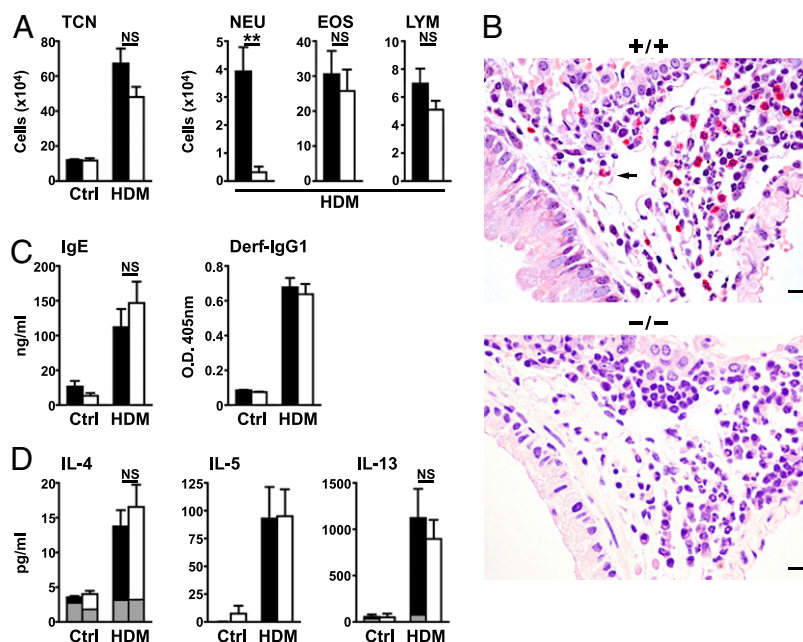
Neutrophils are important effectors of the innate immune response and are activated by Gram-negative LPS. Intranasal administration of LPS in mice was shown to cause neutrophil recruitment and acute lung injury (ALI) (19, 20). Therefore, we investigated the influence of FAST deficiency on neutrophil recruitment to the lung 20 h after a single intranasal challenge with LPS from *E. coli* O5: B55 (200 μ g/kg). Neutrophils represent >90% of the BAL fluid cells in an LPS-induced ALI model. We found that FAST^{-/-} mice are relatively resistant to LPS-induced pulmonary inflammation compared with WT controls. The total number of infiltrating cells and the number of neutrophils in BAL fluid were reduced in FAST^{-/-} mice (2.05 ± 0.39 million neutrophils) compared with WT controls (4.42 ± 0.60 million neutrophils; $p < 0.01$) (Fig. 3A). Histologic analysis confirmed that FAST^{-/-} mice have reduced pulmonary neutrophil accumulation (Fig. 3B). In addition to acute inflammation, vascular leakage is a cardinal feature of ALI. As expected, vascular leakage assessed by total protein in BAL fluid was less in LPS-treated FAST^{-/-} mice compared with WT

controls (0.86 ± 0.09 mg/ml versus 1.44 ± 0.23 mg/ml; $p < 0.05$) (Supplemental Fig. 1A). Also consistent with reduced vascular leakage, the accumulation of extravasated RBCs in FAST^{-/-} BAL fluid was less than that in WT controls (Supplemental Fig. 1B). Thus, the ability of FAST to regulate neutrophil recruitment to the lung is observed in two different models of pulmonary inflammation.

Effect of FAST on neutrophil function

We found that FAST mRNA is expressed in neutrophils (Supplemental Fig. 2). The selective reduction in neutrophil infiltration suggests that the function of FAST^{-/-} neutrophils may be impaired. Flow cytometric analysis revealed that LPS-treated WT and FAST^{-/-} mice have similar percentages and absolute numbers (0.6288 ± 0.1395 K/ μ l versus 0.5588 ± 0.1229 K/ μ l, respectively) of peripheral blood neutrophils (Fig. 4A). Although FAST has been described as a survival factor, in vitro culture of neutrophil populations reveals that the absence of FAST does not affect the levels of spontaneous apoptosis, as determined by staining with Annexin V and 7AAD (Fig. 4B). Thus, the reduced number of neutrophils in inflamed lung is unlikely to result from enhanced apoptosis of cells lacking FAST. We next investigated whether defective chemotaxis of FAST^{-/-} neutrophils might account for the reduced recruitment of neutrophils to the lungs in LPS-treated FAST^{-/-} mice. BM-derived neutrophils from WT or FAST^{-/-} mice were exposed to the potent chemoattractant LTB₄ in a chemotactic chamber (EZ-TAXIScan), and single motile cells were tracked for 20 min with frames taken every 30 s. The cell paths were charted, and average cell speed and directionality were quantified (Fig. 5A). Surprisingly, FAST^{-/-} neutrophils moved significantly faster than did WT neutrophils (12.75 ± 0.56 μ m/min versus 10.34 ± 0.39 μ m/min; $p < 0.001$) (Fig. 5B). The directionality and upward directionality of WT and FAST^{-/-} neutrophils were not significantly different (Fig. 5B). Similar results were obtained using fMLP instead of LTB₄ (Supplemental Fig. 3). Our results indicate that FAST^{-/-} neutrophils are equally as capable as WT neutrophils of chemotaxing and reaching their final target. Thus, the reduced number of pulmonary neutrophils in FAST^{-/-} mice is probably not due to defective neutrophil survival or chemotaxis.

FIGURE 2. Lung inflammation in HDM-treated WT and FAST^{-/-} mice. **A**, Total and differential cell counts in BAL fluid. EOS, eosinophils; LYM, lymphocytes; NEU, neutrophils; TCN, total cell number. ** $p < 0.01$. **B**, Histological examination of inflammation around the bronchovascular bundles. Neutrophils (red-stained cells; arrow) were detected with chloroacetate esterase substrate (original magnification $\times 40$). Scale bar, 10 μ m. **C**, Serum concentration of total IgE and HDM-specific IgG1 Ab titers. **D**, Cytokine profile of splenocytes harvested from mice 24 h after final HDM exposure. Supernatants were collected after 72 h in vitro culture in the absence (gray bars) or presence of HDM (20 μ g/ml). Control (Ctrl) WT and FAST^{-/-} mice were challenged with sterile saline. Means \pm SEM are shown ($n = 10$). Black bars represent WT mice, and white bars represent FAST^{-/-} mice.



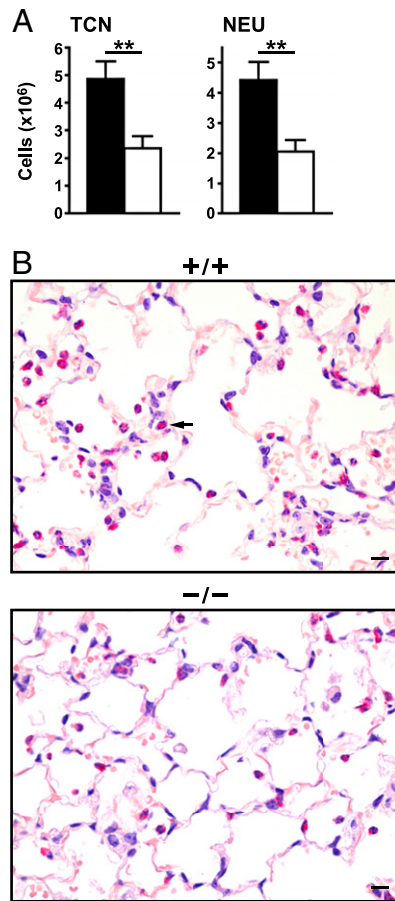


FIGURE 3. Lung inflammation in LPS-treated WT and FAST^{-/-} mice. *A*, Total and neutrophil counts in BAL fluid from LPS-treated WT (black bars) or FAST^{-/-} (white bars) mice ($n = 10$ for each group). $**p < 0.01$. *B*, Histological examination of lung parenchyma and alveolar space in LPS-treated WT and FAST^{-/-} mice. Neutrophils (red-stained cells; arrow) were detected with chloroacetate esterase substrate (original magnification $\times 40$). Scale bar, 10 μ m.

We also compared the response of WT and FAST^{-/-} neutrophils to zymosan particles. The phagocytosis of zymosan particles and the production of reactive oxygen species were similar in WT and FAST^{-/-} neutrophils (Supplemental Fig. 4). Thus, FAST does not seem to affect the survival, chemotaxis, or phagocytic or killing activity of neutrophils.

FAST exerts its proinflammatory effects within the lung

We next compared the ability of fluorescently labeled WT or FAST^{-/-} BM-derived neutrophils to migrate to the lungs of LPS-treated WT or FAST^{-/-} mice. Equivalent amounts of CFSE-labeled WT neutrophils and TRITC-labeled FAST^{-/-} neutrophils were injected i.v. into WT and FAST^{-/-} mice 6 h after intranasal LPS challenge. Two hours later, mice were sacrificed, and BAL fluid was analyzed using flow cytometry. Homing index was calculated as the ratio of CFSE⁺/TRITC⁺ cells. The homing index was very close to 1 in all experiments (Fig. 6A shows representative experiments), indicating that WT CFSE⁺ cells and FAST^{-/-} TRITC⁺ cells have a similar ability to migrate to lungs of LPS-treated WT or FAST^{-/-} mice. We next calculated the absolute number of fluorescently labeled cells in BAL fluid of LPS-treated WT and FAST^{-/-} mice. In the BAL fluid of LPS-treated FAST^{-/-} recipient mice, the absolute numbers of WT CFSE⁺ cells (3117 ± 606.7 cells) and FAST^{-/-} TRITC⁺ cells (3050 ± 632.5 cells) were reduced compared with the absolute numbers of WT CFSE⁺ cells (6188 ± 1034 cells; $p < 0.05$) and FAST^{-/-} TRITC⁺ cells

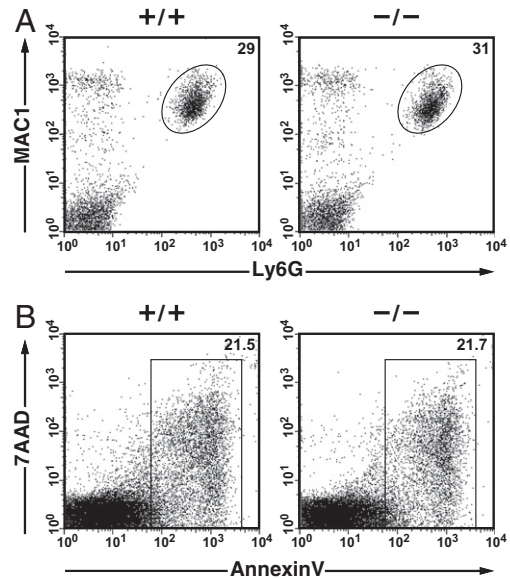


FIGURE 4. *A*, Representative FACS analysis of whole blood taken from LPS-treated mice. Neutrophils were stained with Abs to Mac-1 and Ly6G. *B*, FACS analysis of neutrophil spontaneous death. BM neutrophils were cultured in RPMI 1640 medium containing 10% FBS at a density of 2×10^6 cells/ml for 24 h. Apoptotic cells were detected by Annexin V-FITC and 7AAD staining.

(6188 ± 1034 cells; $p < 0.05$) in LPS-treated WT mice (Fig. 6B). These results indicate that the reduced recruitment of neutrophils to FAST^{-/-} lung is not due to defective neutrophil function and that FAST exerts its proinflammatory effects within the lung.

To determine the basis for the reduced neutrophil recruitment in FAST^{-/-} mice, we quantified proinflammatory cytokines and chemoattractants in BAL fluid. BAL fluid from LPS-treated FAST^{-/-} mice contains significantly less TNF- α (286.2 ± 67.35 pg/ml versus 788.0 ± 141.8 pg/ml; $p < 0.01$), IL-6 (404.8 ± 95.49 pg/ml versus 859.0 ± 148.7 pg/ml; $p < 0.05$), IL-23 (9.929 ± 3.669 pg/ml versus 45.62 ± 7.814 pg/ml; $p < 0.001$), MIP-2 (97.92 ± 10.68 pg/ml versus 201.2 ± 30.88 ; $p < 0.01$), and KC (197.5 ± 43.26 pg/ml versus 385.4 ± 30.29 pg/ml; $p < 0.01$) than LPS-treated WT controls (Fig. 6C). In contrast, levels of G-CSF, IL-1 β , and MCP-1 in BAL fluid were similar in both groups of mice. IL-17A was below the detection limit (0.78 pg/ml) in BAL fluid of mice of both genotypes. These results suggest that FAST mediates its proinflammatory effects within the lung parenchyma by promoting the production of proinflammatory cytokines and chemoattractants.

FAST expression in lung resident cells of hematopoietic origin is necessary for neutrophil recruitment to the lung

We next investigated the relative contribution of hematopoietic and nonhematopoietic cells to the reduced proinflammatory cytokine and chemokine production in the lung parenchyma of LPS-treated FAST^{-/-} mice. We created chimeras and controls by transferring BM between WT and FAST^{-/-} mice. Engraftment efficiency was monitored by WBC counts and PCR (Supplemental Fig. 5). In all groups, mice were assessed 20 h after LPS inhalation. Consistent with the findings in FAST^{-/-} mice, FAST^{-/-}→FAST^{-/-} mice (where FAST^{-/-} BM was injected into irradiated FAST^{-/-} mice) had significantly fewer neutrophils (1.435 ± 0.2607 million cells) in BAL fluid than WT→WT mice (3.433 ± 0.4740 million cells; $p < 0.01$) (Fig. 7). In WT→FAST^{-/-} chimeric mice, the number of neutrophils in the BAL fluid was similar to that of WT→WT mice (Fig. 7). These results indicate that nonhematopoietic cells

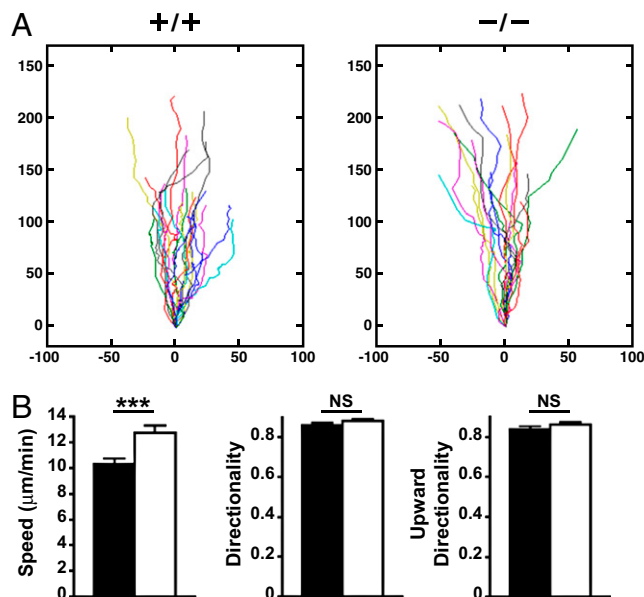


FIGURE 5. Chemotaxis in FAST^{-/-} neutrophils. BM neutrophils isolated from WT and FAST^{-/-} mice were exposed to 100 nM LTB₄ in a chemotactic chamber EZ-TAXIScan, and single motile cells were tracked for 20 min with frames taken every 30 s. *A*, Cell paths are shown starting at each origin. Units in *x*- and *y*-axes, μm. *B*, Parameters of motility, such as average migration speed, directionality, and upward directionality, are shown. Results are mean ± SEM of *n* = 20 cells for each group from three movie sequences. Black bars represent WT mice, and white bars represent FAST^{-/-} mice. ****p* < 0.001.

are not a major determinant of the reduced inflammatory response in FAST^{-/-} lungs. In contrast, FAST^{-/-}→WT chimeric mice showed a reduction in the number of neutrophils in the BAL fluid (1.874 ± 0.4698 million cells; *p* < 0.05) that was similar to that of FAST^{-/-}→FAST^{-/-} controls. As shown in Supplemental Fig. 6, TNF-α levels in the BAL fluid samples correlated well with neutrophil numbers. These results suggest that FAST expression in lung resident cells of hematopoietic origin (alveolar macrophages and/or dendritic cells [DCs]) is required to produce the inflammatory cytokines and/or neutrophil chemoattractants that mediate pulmonary inflammation.

Discussion

FAST is a serine/threonine phosphoprotein that interacts with the adenine/uridine-rich element RNA-binding protein TIA-1. TIA-1 is a translational repressor that inhibits the production of multiple proinflammatory mediators, including TNF-α, IL-1β and -6, cyclooxygenase 2, and matrix metalloproteinase 13 (8, 10, 21). Mice lacking TIA-1 overexpress these inflammatory mediators and develop spontaneous arthritis, a manifestation of a general inflammatory diathesis (22). Because FAST antagonizes TIA-1-induced translational repression, we predicted that FAST^{-/-} mice would produce less inflammatory mediators and exhibit less inflammation than WT controls. Consistent with this prediction, we found that FAST^{-/-} mice are resistant to immune-mediated pulmonary inflammation.

In a well-established model of HDM-induced allergic pulmonary inflammation, we found that FAST^{-/-} mice and WT controls showed similar eosinophilic airway inflammation. The production of Th2 cytokines that drive eosinophilia in this model was also similar in WT and FAST^{-/-} mice. Interestingly, we found a striking reduction in neutrophils in pulmonary tissue and BAL fluids of HDM-treated FAST^{-/-} mice. Neutrophils represent a small per-

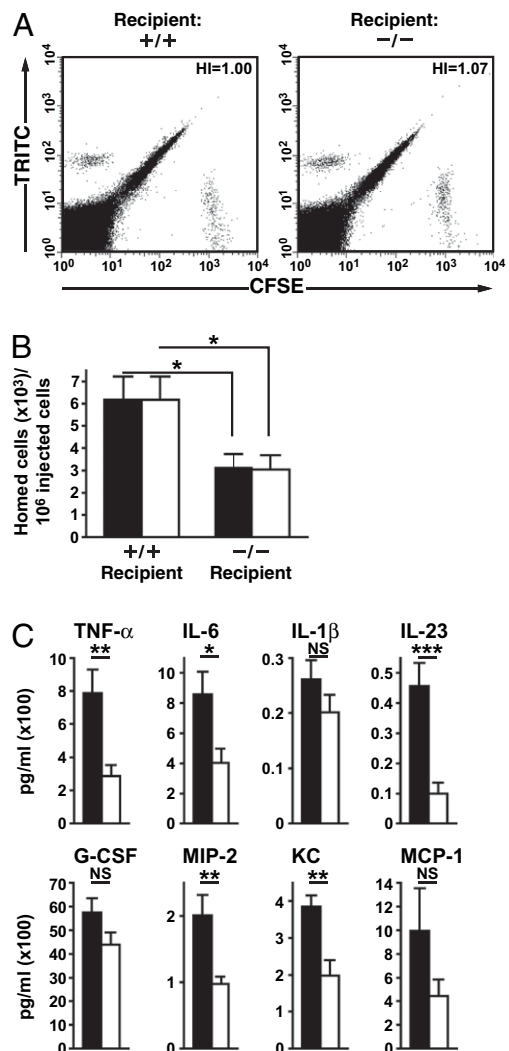


FIGURE 6. Recruitment of adoptively transferred neutrophils in LPS-treated WT and FAST^{-/-} mice. A mixture of equal numbers of CFSE-labeled WT neutrophils and TRITC-labeled FAST^{-/-} neutrophils was injected i.v. into WT and FAST^{-/-} mice that had been challenged with LPS for 6 h. *A*, FACS analysis of the recipient BAL fluid 2 h after the injection. The homing index (HI) represents the ratio of CFSE⁺/TRITC⁺ cells. *B*, Absolute number of adoptively transferred neutrophils recruited to the alveolar space. Data shown are means ± SEM for six mice. *C*, Levels of several cytokines and chemoattractants in BAL fluid of mice 20 h after intranasal instillation of LPS. Means ± SEM are shown (*n* = 8–10). Black bars represent WT mice, and white bars represent FAST^{-/-} mice. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

centage of the overall inflammatory cell response in this model of asthma. Patients with severe and fatal asthma have increased numbers of neutrophils in the airways (23). Several reports have suggested an important role for neutrophils in airway remodeling. Asthmatic patients have greater numbers of subepithelial neutrophils positive for TGF-β than do normal control subjects (24). TGF-β has an important role in tissue repair and fibrosis, and its levels may correlate with the thickness of subepithelial basement membrane (as an indicator of airway fibrosis) (25). In addition, neutrophil-derived oxidative stress is known to cause mucin MUC5AC synthesis via ligand-independent epidermal growth factor receptor (26). Increased production of mucin MUC5AC causes goblet cell hyperplasia (27). In HDM-treated FAST^{-/-} mice, there was a trend toward reduced numbers of goblet cells (detected by Alcian blue/periodic acid-Schiff staining) compared

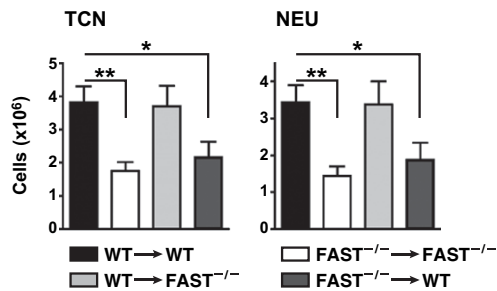


FIGURE 7. Lung inflammation in LPS-treated chimeric mice. Chimeric mice preparation denoted as BM donor genotype→recipient genotype. The resulting four groups of mice were intranasally treated with LPS; 20 h later, total and neutrophil counts in BAL fluid were assessed. Means \pm SEM are shown ($n = 6-8$). Black bars represent WT mice, and white bars represent FAST^{-/-} mice. * $p < 0.05$; ** $p < 0.01$.

with WT controls, but the difference was not statistically significant ($31.14 \pm 3.885/\text{mm}$ [$n = 30$] versus $40.56 \pm 3.682/\text{mm}$ [$n = 29$]; mm of perimeter of the bronchus). The role of neutrophils in mouse models of asthma is largely unexplored. In these models, neutrophils increase transiently in the BAL fluid (28) as a result of challenge with a large allergen bolus.

The neutrophil phenotype prompted us to examine the role of FAST in the development of LPS-mediated ALI. Recruitment of neutrophils into the lung is a pathological hallmark of this model of ALI (29). TLR4 is the most important cellular receptor for LPS and is essential for LPS-induced neutrophil migration into the lung, as shown by the absence of a response in TLR4-deficient mice (30–32). In lung tissue, nonhematopoietic radioresistant cells (such as epithelial and endothelial cells) and hematopoietic cells (such as alveolar, lung macrophages, and DCs) are instrumental in inducing innate responses to LPS (33–37). In this report, we conclude that FAST expression on lung cells of hematopoietic origin is crucial in mediating LPS-induced neutrophil migration to the lung. The reduction in neutrophil infiltration in the lungs of LPS-treated FAST^{-/-} mice is accompanied by reduced levels of proinflammatory cytokines (e.g., TNF- α and IL-6 and -23) and neutrophil chemoattractants (e.g., MIP-2 and KC) in the BAL fluids. Interestingly, KC and MIP-2 are the chemoattractants that recruit neutrophils to the lung in mice challenged with HDM Ag (38). These same chemokines are likely to contribute to neutrophil infiltration in the LPS model as well. Although the inhibition of neutrophil recruitment is more pronounced in the HDM model compared with the LPS model, this may reflect differences in the delivered dose of LPS rather than a role for FAST in the adaptive immune response to HDM Ag. The finding that FAST^{-/-} mice develop pulmonary inflammation in the absence of neutrophilia may allow us to determine the importance of neutrophils for the development of airway hyperreactivity, a phenomenon that can be dissociated from pulmonary inflammation.

Alveolar macrophages are lung resident cells of hematopoietic origin that play a very important role in the development of ALI. Activated pulmonary macrophages release the cytokines TNF- α and IL-1 β , -6, and -8, as well as the chemokines MCP-1 and MIP-1 β and -2 (39). Depletion of alveolar macrophages by clodronate-liposomes resulted in decreased neutrophilic influx and pulmonary TNF- α production following exposure to aerosolized LPS (34). Numerous studies have reported varying effects of macrophage depletion on the development of neutrophilic lung injury in infectious models of lung inflammation with Gram-negative bacteria. Although some investigators found decreased expression of

TNF- α and MIP-2 in association with decreased neutrophil influx in *Pseudomonas aeruginosa* pneumonia (40), other investigators described increased neutrophil influx into the lungs following inoculation with *Klebsiella pneumoniae* (41). These varying effects on neutrophil influx are likely due to impaired clearance of bacteria in the absence of alveolar macrophages that is a persistent stimulus for neutrophil influx. It will be interesting to characterize the role of FAST in different infectious models of lung inflammation.

Lung-resident DCs participate in innate and adaptive immunity in the lung. As a result of activation through TLRs, DCs release cytokines (e.g., TNF- α and IL-1 β and -6) and chemokines (e.g., MIP-1 α and -2) (42) that promote acute lung inflammation. DCs are the most potent APCs and generate robust Th1 and Th2 immune responses (43). DCs bridge innate and adaptive responses in the model of HDM-induced allergic pulmonary inflammation. An emerging theme in the field of lung immunology is that structural cells of the airways, such as epithelial cells, endothelial cells, and fibroblasts, produce activating cytokines that determine the quantity and quality of the lung immune response. It was recently reported that TLR4 triggering on epithelium plays a central role in controlling the function of lung DCs through the release of innate cytokines (GM-CSF, IL-25 and -33, and thymic stromal lymphopoietin) that promote DC maturation and boost Th2 cytokine production (33, 44–47). The HDM extracts are known to contain LPS (48, 49). WT→TLR4^{-/-} chimeric mice failed to develop the salient features of allergic inflammation, such as airway eosinophilia, goblet cell hyperplasia, and peribronchial and perivascular inflammation, following exposure to HDM (33). Our study showed that HDM-treated FAST^{-/-} mice developed eosinophilic airway inflammation similar to that of WT controls, suggesting that FAST is not a major determinant of the activation of the epithelium-DC axis by LPS.

There are many ways in which the absence of FAST could produce the observed phenotype; the translation of one or more of these transcripts could be enhanced in the absence of FAST-mediated repression of TIA-1. This regulation may occur in a cell-type-restricted manner, because TIA-1 represses the translation of TNF- α in macrophages but not in T cells. Cells within the inflamed lung that may produce neutrophil chemoattractants include alveolar macrophages, pulmonary DCs, infiltrating T cells, or pulmonary epithelial cells. The reduced expression of a pivotal cytokine (e.g., TNF- α or IL-6) could indirectly reduce the expression of the other mediators by dampening inflammation. Further studies will be required to identify cells that require FAST for the production of inflammatory mediators. Also, the absence of FAST may prevent the survival of key lung-resident cells of hematopoietic origin, or altered splicing of Fas receptor mRNA could increase the production of soluble Fas. This could increase the survival of immunomodulatory cells to inhibit pulmonary inflammation. In addition, it is known that alternate splicing of key signaling molecules in TLR cascades dramatically alters the signaling capacity of inflammatory cells (50). Because more than one of these mechanisms may conspire to dampen pulmonary inflammation, nonbiased analysis of inflammatory mediator production and infiltrating immune cells will be required to shed light on the mechanism by which FAST promotes immune-mediated pulmonary inflammation.

The finding that FAST is overexpressed in peripheral blood cells from patients with many immune-mediated inflammatory diseases suggests that FAST may be an important mediator of this process. Future studies designed to determine the mechanism by which FAST promotes inflammation could identify targets for a new class of anti-inflammatory drugs.

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Disclosures

The authors have no financial conflicts of interest.

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