NIH Public Access
Author Manuscript
J Immunol. Author manuscript; available in PMC 2011 May 1.

Published in final edited form as:
J Immunol. 2010 May 1; 184(9): 5325–5332. doi:10.4049/jimmunol.1000104.

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**[*]

[*] Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, and Department of Medicine, Harvard Medical School, Boston, MA 02115 [†] Department of Clinical Pathophysiology and Experimental Medicine, University of Naples "Federico II", Naples, Italy [‡] Division of Immunology, Children's Hospital and Department of Pediatrics, Harvard Medical School, Boston, MA 02115 [§] Theodor Kocher Institute, University of Bern, 3012 Bern, Switzerland [¶] Department of Lab Medicine and Joint Program in Transfusion Medicine, Children's Hospital and Department of Pathology, Boston, MA 02115 [||] Unidad de Investigación, Hospital Clínico Universitario de Valladolid, 47005 Valladolid, Spain

Abstract

We have generated Fas activated serine threonine phosphoprotein-deficient mice ($FAST^{-/-}$) to study the in vivo role of FAST in immune system function. In a model of house dust mite (HDM)-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 to wild type controls. This is accompanied by reduced concentrations of cytokines (TNF-α, IL-6 and IL-23) and chemoattractants (MIP-2 and KC) in bronchoalveolar lavage fluids. As $FAST^{-/-}$ 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 pro-inflammatory factor that modulates the function of lung resident hematopoietic cells to promote neutrophil recruitment and pulmonary inflammation.

INTRODUCTION

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 antigen-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 co-localized in cytoplasmic stress granules, a compartment that helps to re-program protein expression to promote survival under adverse environmental

Correspondence: Paul Anderson, M.D., Ph.D., Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Smith 652, One Jimmy Fund Way, Boston, MA 02115, Telephone: 617-525-1202, FAX: 617-525-1310, panderson@rics.bwh.harvard.edu.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

conditions. Thus, FAST and TIA-1 are functional antagonists that help 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 the Fas receptor 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 soluble and membrane-associated Fas receptor, 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 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 peripheral blood mononuclear cells 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 inflammatory diathesis that is manifest by spontaneous arthritis (10). Here 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) 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), was cloned into pBluescript II KS+ (pBS, Stratagene). This modified pBS 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), and cells were then selected in medium containing 200 μg/ml G418 and 1 μM ganciclovir. Correctly targeted clones were identified by Southern blotting

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

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, Jackson Lab) 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 #000 664, Jackson) 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 of HDM extract (Greer Laboratories, Lenoir, NC) in 20 μl of sterile saline for 2 days 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 mg/Kg LPS from Escherichia coli 055:B5 (Sigma) by intranasal instillation, and the mice were killed 20 h later for BAL and blood collection.

BAL

Tracheas were canulated with an 18-gauge angiocath. Lungs were lavaged three times with 0.7 ml of cold PBS containing 0.5 mM EDTA. The cells were counted, cytospun and stained with Diff-Quick (Dade Behring) 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

Red blood cell-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 week 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 E9.5 embryos.

Cytokine and Ig measurements

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

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

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 positive 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). 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 positive, 7AAD negative) from late apoptotic cells (annexin V-FITC positive, 7AAD-bright). Stained cells were acquired on a FACSCalibur flow cytometer (Becton Dickinson) and data processed by FlowJo software (Tree Star Inc.).

Blood counts

EDTA anti-coagulated 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 of chemoattractant (100 nM LTB4 or 1 μM fMLP) was put into another contra-hole. The chamber was incubated 20 min at 37°C. To count the migrated cells in each channel, images of the cells in each channel were captured at 30 sec intervals using the 10x lens on a Discovery Screening System (Universal Imaging Corporation, Dowingtown, PA). *(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 the cell moved. Upward directionality was calculated by dividing the straight-line distance the cell moved in the upward direction divided by total curve-line distance 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), 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 ratio 1:1 and 2×10^6 cells were injected i.v. into WT and $FAST^{-/-}$ mice 6 h after being challenged with 200 mg/Kg LPS intranasally. Two hours after injection, recipient mice were killed and BAL-cell suspensions were analyzed by flow cytometry and the percentages of CFSE+ and TRITC+ were determined. The homing index was calculated as the ratio of CFSE+ to TRITC+ cells in the BAL fluid divided by the ratio of CFSE+ to TRITC+ cells in the input.

Assay for production of reactive oxygen species

Production of reactive oxygen species (ROS) was assayed by luminol-enhanced chemiluminescence. Mice 8–12 weeks old were injected intraperitoneally with 3%

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

thioglycollate broth (2 ml) 4 h prior to peritoneal lavage with RPMI. The proportion of neutrophils in the peritoneal lavage was >90% judged by light microscopy of Diff-Quick stained cytospin preparations. The assay mixture (0.2 ml) contained 200 μM luminol and 2×10^5 neutrophils in HBSS. Neutrophils were activated with zymosan (30 particles per cell) (Sigma Chemical Co.) in HBSS or HBSS as a control. Chemiluminescence was measured at showed intervals with a luminometer (Monolight 2010, Analytical Luminescence Laboratory, Ann Arbor, MI) and expressed as relative luciferase unit (RLU) per 1×10^4 cells.

Generation of chimeric mice

Three-week old recipient mice were lethally irradiated in 2 doses of 600 rads each (separated by 4 h). BM from donor mice was harvested from both femora and tibiae, and approximately 6×10^6 cells in 0.2 ml were injected i.v. into recipient mice. BM transplantation was performed in 4 groups of mice: (a) BM from $FAST^{-/-}$ into WT ($FAST^{-/-}\rightarrow$WT chimeric, expressing FAST on nonhematopoietic cells only); (b) BM from WT into $FAST^{-/-}$ mice (WT→$FAST^{-/-}$ chimeric, expressing FAST on hematopoietic cells ony); (c) BM from $FAST^{-/-}$ to $FAST^{-/-}$ ($FAST^{-/-}\rightarrow FAST^{-/-}$); and (d) BM from WT to WT (WT→WT). Mice in the latter 2 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 either a 384 bp fragment on the WT allele with the MSG128/MSG129 primer pair, or a 669 bp fragment on the KO allele with the MSG105/MSG133 primer pair (primer sequences are provided in Supplemental Table I).

Northern blot, immunoblot, and real time quantitative PCR analysis

FAST transcript and protein expression in MEFs were evaluated by Northern blot using a FAST complementary DNA fragment (nucleotides 1369-1621 of GenBank accession no. BC013547) and by immunoblot using goat polyclonal anti-FAST (Santa Cruz). Relative mRNA expression levels of FAST in lung and neutrophils were measured by SYBR Green based real time quantitative PCR assay. GAPDH, β-actin, Beta-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); MSG323/MSG324 (for hypoxanthine phosphoribosyltransferase 1) (primer sequences are provided in Supplemental Table I). Primer pairs for Beta-2 microglobulin and 18S were purchased from Superarray. Data were analyzed using GeNorm software.

Statistics

All analyses were performed using Prism software (GraphPad). Data are expressed as mean ± SEM and were analyzed by using either one-way ANOVA with Bonferroni correction or the unpaired Student's t test, as appropriate. Significance is indicated by an asterisk (*) on the figures when $P < 0.05$, by two asterisks (**) when $P < 0.01$, and with three asterisks (***) when $P < 0.001$.

RESULTS

Generation of $FAST^{-/-}$ mice

The genomic FAST locus comprises 10 exons spanning 4.3 kb (Figure 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

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

genomic DNA. This vector was transfected into W4/129S6 ES cells for selection of stable integrants that were identified using Southern blotting (Figure 1B) and PCR (Figure 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 (Figure 1D) or protein (Figure 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, Mac-1) or lymphoid (CD45R/B220, CD3, CD4, CD8) lineages (data not shown).

FAST promotes HDM-induced Allergic Pulmonary Inflammation

The finding that FAST is overexpressed in peripheral blood mononuclear cells 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 antigen model of allergic pulmonary inflammation because it utilizes a clinically relevant allergen with endogenous adjuvants that break tolerance across the mucosal barrier (17,18). Mice were exposed to intranasal HDM extract 2 days per week for 3 weeks, a protocol that results in a Th2-associated 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 approximately 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 (Figure 2A), compositional analysis revealed the virtual absence of neutrophils in $FAST^{-/-}$ BAL fluid ($0.32 \times 10^4 \pm 0.19 \times 10^4$ cells vs $3.91 \times 10^4 \pm 0.35 \times 10^4$ cells, $P < 0.01$) (Figure 2A). Histological analysis of the lungs confirmed the virtual absence of neutrophils in over 95% of the bronchovascular bundles analyzed (Figure 2B). In both WT and $FAST^{-/-}$ mice, HDM exposure increased total serum IgE and HDM-specific serum IgG1 to similar levels indicating an intact immune response (Figure 2C). Culture of WT and $FAST^{-/-}$ splenocytes from HDM-challenged mice in the presence of HDM extract (20 μg/mL) for 4 days resulted in the secretion of similar amounts of IL-4, IL-5, and IL-13 (Figure 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 has been shown to cause neutrophil recruitment and acute lung injury (ALI) (19,20). We therefore investigated the influence of FAST deficiency on neutrophil recruitment to the lung 20 h after a single intranasal challenge with LPS from Escherichia coli 05:B55 (200 μg/Kg). Neutrophils represent over 90% of the BAL fluid cells in LPS-induced ALI model. We found that $FAST^{-/-}$ mice are relatively resistant to LPS-induced pulmonary inflammation compared to WT controls. Both the total number of infiltrating cells and the number of neutrophils in BAL fluid are reduced in $FAST^{-/-}$ mice (2.05 ± 0.39 million neutrophils) compared to WT controls (4.42 ± 0.60 million neutrophils, $P < 0.01$) (Figure 3A). Histologic analysis confirms that $FAST^{-/-}$ mice have reduced pulmonary neutrophil accumulation (Figure 3B).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Another cardinal feature of ALI besides acute inflammation is vascular leakage. As expected, vascular leakage assessed by total protein in BAL fluid is less in LPS-treated FAST$^{-/-}$ mice compared to WT controls (0.86 ± 0.09 mg/ml vs 1.44 ± 0.23 mg/ml, $P < 0.05$) (Supplemental Figure 1A). Also consistent with reduced vascular leakage, the accumulation of extravasated red blood cells in FAST$^{-/-}$ BAL fluid was less than that in WT controls (Supplemental Figure 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 have found that FAST mRNA is expressed in neutrophils (Supplemental Figure 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/ml vs 0.5588 ± 0.1229 K/ml, respectively) of peripheral blood neutrophils (Figure 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 levels of spontaneous apoptosis as determined by staining with Annexin-V and 7AAD (Figure 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 either WT or FAST$^{-/-}$ mice were exposed to the potent chemoattractant leukotriene B4 (LTB4) in a chemotactic chamber (EZ-TAXIScan) and single motile cells were tracked for 20 minutes with frames taken every 30 seconds. The cell paths were charted and average cell speed and directionality were quantified (Figure 5A). Surprisingly, FAST$^{-/-}$ neutrophils move significantly faster than WT neutrophils (12.75 ± 0.56 mm/sec vs 10.34 ± 0.39 mm/sec, $P < 0.001$) (Figure 5B). The directionality and upward directionality of WT and FAST$^{-/-}$ neutrophils are not significantly different (Figure 5B). Similar results were obtained using fMLP instead of LTB4 (Supplemental Figure 3). Our results indicate that FAST$^{-/-}$ neutrophils are equally capable of chemotaxing and reaching their final target, when compared with WT neutrophils. Thus, the reduced number of pulmonary neutrophils in FAST$^{-/-}$ mice is unlikely to be due to defective neutrophil survival or chemotaxis.

We also compared the response of WT and FAST$^{-/-}$ neutrophils to zymosan particles. Both the phagocytosis of zymosan particles and the production of reactive oxygen species (ROS) was found to be similar in WT and FAST$^{-/-}$ neutrophils (Supplemental Figure 4). Thus, FAST does not appear to affect the survival, chemotaxis, phagocytic or killing activity of neutrophils.

FAST exerts its pro-inflammatory 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+ to TRITC+ cells. The homing index was very close to 1 in all experiments (Figure 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 to the absolute

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

numbers of WT CFSE+ cells (6188 ± 1034 cells, $P < 0.05$) and FAST$^{-/-}$ TRITC+ cells (6188 ± 1034 cells, $P < 0.05$) in LPS-treated WT mice (Figure 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 pro-inflammatory effects within the lung.

To determine the basis for the reduced neutrophil recruitment in FAST$^{-/-}$ mice, we quantified pro-inflammatory cytokines and chemoattractants in BAL fluid. BAL fluid from LPS-treated FAST$^{-/-}$ mice contains significantly less TNF-α (286.2 ± 67.35 pg/ml vs 788.0 ± 141.8 pg/ml, $P < 0.01$), IL-6 (404.8 ± 95.49 pg/ml vs 859.0 ± 148.7 pg/ml, $P < 0.05$), IL-23 (9.929 ± 3.669 pg/ml vs 45.62 ± 7.814 pg/ml, $P < 0.001$), MIP-2 (97.92 ± 10.68 pg/ml vs 201.2 ± 30.88, $P < 0.01$) and KC (keratinocyte chemoattractant) (197.5 ± 43.26 pg/ml vs 385.4 ± 30.29 pg/ml, $P < 0.01$) than LPS-treated WT controls (Figure 6C). In contrast, levels of G-CSF, IL1-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 pg/ml) in BAL fluid of mice of both genotypes. These results suggest that FAST mediates its pro-inflammatory effects within the lung parenchyma by promoting the production of pro-inflammatory 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 non-hematopoietic cells to the reduced pro-inflammatory 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 white blood cell counts and PCR (Supplemental Figure 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 less neutrophils (1.435 ± 0.2607 million cells) in BAL fluid than WT→WT mice (3.433 ± 0.4740 million cells, $P < 0.01$) (Figure 7). In WT→FAST$^{-/-}$ chimeric mice, the number of neutrophils in the BAL fluid was similar to that of WT→WT mice (Figure 7). These results indicate that non-hematopoietic cells are not a major determinant of the reduced inflammatory response in FAST$^{-/-}$ lungs. In contrast, FAST$^{-/-}$ →WT chimeric mice showed a reduction of 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 Figure 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) 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 pro-inflammatory mediators including TNF-α, IL-1β, IL-6, cyclooxygenase 2 and matrix metalloproteinase 13 (8,10,21). Mice lacking TIA-1 over-express these inflammatory mediators and develop spontaneous arthritis, a manifestation of a general inflammatory diathesis (22). As 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 have 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

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

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 both pulmonary tissue and BAL fluids of HDM-treated FAST$^{-/-}$ mice. Neutrophils represent a small percentage 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 transforming growth factor β than normal control subjects (24).

Transforming growth factor 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 towards reduced numbers of goblet cells (detected by alcian blue/periodic acid-Schiff staining) compared to WT controls, but the difference was not statistically significant (31.14 ± 3.885/mm n=30 vs 40.56 ± 3.682/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 a 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 acute lung injury (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, both non-hematopoietic radioresistant cells (such as epithelial and endothelial cells) and hematopoietic cells (such as alveolar, lung macrophages and dendritic cells) 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 pro-inflammatory cytokines (e.g, TNF-α, IL-6, IL-23) and neutrophil chemoattractants (e.g., MIP-2, KC) in the BAL fluids. Interestingly, KC and MIP-2 are the chemoattractants that recruit neutrophils to the lung in mice challenged with HDM antigen (38). These same chemokines are likely to contribute to neutrophil infiltration in the LPS model as well. Although the inhibition of neutrophil recruitement is more pronounced in the HDM model compare with the LPS model, the may reflect differences in the delivered dose of LPS rather than a role for FAST in the adaptive immune response to HDM antigen. 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 hyper-reactivity, 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, IL-1β, IL-6, IL-8 as well as the chemokines MCP-1, MIP-1β and MIP-2 (39). Depletion of alveolar macrophages by clodronate-liposomes resulted in decreased neutrophilic influx and pulmonary TNF-α production following exposure to aerolized LPS (34). Numerous studies have reported varying effects of macrophage depletion on the development of neutrophilic lung injury on infectious models of lung inflammation with gram negative bacteria. While some investigators have found decreased expression of TNF-α and MIP-2 in association with decreased neutrophil influx in Pseudomonas aeruginosa pneumonia (40), other investigators have described increased neutrophil influx into the

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

lungs following inoculation with Klebsiella pneumonia (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 dendritic cells (DC) participate in both innate and adaptive immunity in the lung. As a result of activation through TLRs, DCs release cytokines (e.g., TNF-α, IL-1β, IL-6) as well as chemokines (e.g.,MIP-1α and MIP-2) (42) that promote acute lung infammation. 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 infammation. 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 has been recently reported that TLR4 triggering on epithelium plays a central role in controlling the function of lung DCs through release of innate cytokines (GM-CSF, IL-25, IL-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 shows HDM-treated FAST$^{-/-}$ mice develop eosinophilic airway inflammation similar to that of WT controls suggesting that FAST is not a major determinant of the activation of the epithelium-DCs axis by LPS.

There are many ways in which the absence of FAST could produce the observed phenotype: 1) 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 as 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 dendritic cells, infiltrating T cells, or pulmonary epithelial cells. 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. 2) The absence of FAST may prevent the survival of key lung resident cells of hematopoietic origin. 3) Altered splicing of Fas receptor mRNA could increase the production of soluble Fas receptor. 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 alter the signaling capacity of inflammatory cells (50). As more than one of these mechanisms may conspire to dampen pulmonary inflammation, non-biased analysis of inflammatory mediator production as well as 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Acknowledgments

This work was supported by RO1 AR051472 from the NIH (PA).

We acknowledge Kirsten Rhee and Samantha Stewart for technical support.

Abbreviations used in this study

7AAD	7-aminoactinomycin D
ALI	acute lung injury
BAL	bronchoalveolar lavage
BM	bone marrow
DC	dendritic cell
ES	embryonic stem
FAST	Fas activated serine threonine phosphoprotein
KC	keratinocyte chemoattractant
LTB4	leukotriene B4
MEF	mouse embryonic fibroblast
ROS	reactive oxygen species
TIA-1	T-cell intracellular antigen-1
TIAR	TIA-1 related protein
TRITC	tetramethylrhodamine-5-(and-6)-isothiocyanate
WT	wild type

References

1. Li W, Kedersha N, Chen S, Gilks N, Lee G, Anderson P. FAST is a BCL-X(L)-associated mitochondrial protein. Biochem Biophys Res Commun 2004;318:95–102. [PubMed: 15110758]
2. Li W, Simarro M, Kedersha N, Anderson P. FAST is a survival protein that senses mitochondrial stress and modulates TIA-1-regulated changes in protein expression. Mol Cell Biol 2004;24:10718–10732. [PubMed: 15572676]
3. Izquierdo JM, Valcarcel J. Fas-activated serine/threonine kinase (FAST K) synergizes with TIA-1/TIAR proteins to regulate Fas alternative splicing. J Biol Chem 2007;282:1539–1543. [PubMed: 17135269]
4. Simarro M, Mauger D, Rhee K, Pujana MA, Kedersha NL, Yamasaki S, Cusick ME, Vidal M, Garcia-Blanco MA, Anderson P. Fas-activated serine/threonine phosphoprotein (FAST) is a regulator of alternative splicing. Proc Natl Acad Sci U S A 2007;104:11370–11375. [PubMed: 17592127]
5. Bauer A, Kleeff J, Bier M, Wirtz M, Kayed H, Esposito I, Korc M, Hafner M, Hoheisel JD, Friess H. Identification of malignancy factors by analyzing cystic tumors of the pancreas. Pancreatology 2009;9:34–44. [PubMed: 19077453]
6. van Doorn R, van Kester MS, Dijkman R, Vermeer MH, Mulder AA, Szuhai K, Knijnenburg J, Boer JM, Willemze R, Tensen CP. Oncogenomic analysis of mycosis fungoides reveals major differences with Sezary syndrome. Blood 2009;113:127–136. [PubMed: 18832135]
7. Yamasaki S, Stoecklin G, Kedersha N, Simarro M, Anderson P. T-cell intracellular antigen-1 (TIA-1)-induced translational silencing promotes the decay of selected mRNAs. J Biol Chem 2007;282:30070–30077. [PubMed: 17711853]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

8. Dixon DA, Balch GC, Kedersha N, Anderson P, Zimmerman GA, Beauchamp RD, Prescott SM. Regulation of cyclooxygenase-2 expression by the translational silencer TIA-1. J Exp Med 2003;198:475–481. [PubMed: 12885872]
9. Lopez de Silanes I, Galban S, Martindale JL, Yang X, Mazan-Mamczarz K, Indig FE, Falco G, Zhan M, Gorospe M. Identification and functional outcome of mRNAs associated with RNA-binding protein TIA-1. Mol Cell Biol 2005;25:9520–9531. [PubMed: 16227602]
10. Piecyk M, Wax S, Beck AR, Kedersha N, Gupta M, Maritim B, Chen S, Gueydan C, Kruys V, Streuli M, Anderson P. TIA-1 is a translational silencer that selectively regulates the expression of TNF-alpha. Embo J 2000;19:4154–4163. [PubMed: 10921895]
11. Reyes R, Alcalde J, Izquierdo JM. Depletion of T-cell intracellular antigen proteins promotes cell proliferation. Genome Biol 2009;10:R87. [PubMed: 19709424]
12. Brutsche MH I, Brutsche C, Wood P, Brass A, Morrison N, Rattay M, Mogulkoc N, Simler N, Craven M, Custovic A, Egan JJ, Woodcock A. Apoptosis signals in atopy and asthma measured with cDNA arrays. Clin Exp Immunol 2001;123:181–187. [PubMed: 11207646]
13. Maas K, Chan S, Parker J, Slater A, Moore J, Olsen N, Aune TM. Cutting edge: molecular portrait of human autoimmune disease. J Immunol 2002;169:5–9. [PubMed: 12077221]
14. Subramanian KK, Jia Y, Zhu D, Simms BT, Jo H, Hattori H, You J, Mizgerd JP, Luo HR. Tumor suppressor PTEN is a physiologic suppressor of chemoattractant-mediated neutrophil functions. Blood 2007;109:4028–4037. [PubMed: 17202315]
15. Cates EC, Fattouh R, Wattie J, Inman MD, Goncharova S, Coyle AJ, Gutierrez-Ramos JC, Jordana M. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. J Immunol 2004;173:6384–6392. [PubMed: 15528378]
16. Matute-Bello G, Lee JS, Frevert CW, Liles WC, Sutlief S, Ballman K, Wong V, Selk A, Martin TR. Optimal timing to repopulation of resident alveolar macrophages with donor cells following total body irradiation and bone marrow transplantation in mice. J Immunol Methods 2004;292:25–34. [PubMed: 15350509]
17. Johnson JR, Swirski FK, Gajewska BU, Wiley RE, Fattouh R, Pacitto SR, Wong JK, Stampfli MR, Jordana M. Divergent immune responses to house dust mite lead to distinct structural-functional phenotypes. Am J Physiol Lung Cell Mol Physiol 2007;293:L730–739. [PubMed: 17586699]
18. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, Gutierrez-Ramos JC, Ellis R, Inman MD, Jordana M. Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. Am J Respir Crit Care Med 2004;169:378–385. [PubMed: 14597485]
19. Chignard M, Balloy V. Neutrophil recruitment and increased permeability during acute lung injury induced by lipopolysaccharide. Am J Physiol Lung Cell Mol Physiol 2000;279:L1083–1090. [PubMed: 11076798]
20. Hirano S. Migratory responses of PMN after intraperitoneal and intratracheal administration of lipopolysaccharide. Am J Physiol 1996;270:L836–845. [PubMed: 8967519]
21. Yu Q, Cok SJ, Zeng C, Morrison AR. Translational repression of human matrix metalloproteinases-13 by an alternatively spliced form of T-cell-restricted intracellular antigen-related protein (TIAR). J Biol Chem 2003;278:1579–1584. [PubMed: 12426321]
22. Phillips K, Kedersha N, Shen L, Blackshear PJ, Anderson P. Arthritis suppressor genes TIA-1 and TTP dampen the expression of tumor necrosis factor alpha, cyclooxygenase 2, and inflammatory arthritis. Proc Natl Acad Sci U S A 2004;101:2011–2016. [PubMed: 14769925]
23. Sur S, Crotty TB, Kephart GM, Hyma BA, Colby TV, Reed CE, Hunt LW, Gleich GJ. Sudden-onset fatal asthma. A distinct entity with few eosinophils and relatively more neutrophils in the airway submucosa? Am Rev Respir Dis 1993;148:713–719. [PubMed: 8368644]
24. Chu HW, Trudeau JB, Balzar S, Wenzel SE. Peripheral blood and airway tissue expression of transforming growth factor beta by neutrophils in asthmatic subjects and normal control subjects. J Allergy Clin Immunol 2000;106:1115–1123. [PubMed: 11112895]
25. Vignola AM, Chanez P, Chiappara G, Merendino A, Pace E, Rizzo A, la Rocca AM, Bellia V, Bonsignore G, Bousquet J. Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. Am J Respir Crit Care Med 1997;156:591–599. [PubMed: 9279245]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

26. Takeyama K, Dabbagh K, Jeong Shim J, Dao-Pick T, Ueki IF, Nadel JA. Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: role of neutrophils. J Immunol 2000;164:1546–1552. [PubMed: 10640773]
27. Hovenberg HW, Davies JR, Carlstedt I. Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells. Biochem J 1996;318(Pt 1):319–324. [PubMed: 8761488]
28. Taube C, Dakhama A, Takeda K, Nick JA, Gelfand EW. Allergen-specific early neutrophil infiltration after allergen challenge in a murine model. Chest 2003;123:410S–411S. [PubMed: 12629005]
29. Abraham E. Neutrophils and acute lung injury. Crit Care Med 2003;31:S195–199. [PubMed: 12682440]
30. Andonegui G, Goyert SM, Kubes P. Lipopolysaccharide-induced leukocyte-endothelial cell interactions: a role for CD14 versus toll-like receptor 4 within microvessels. J Immunol 2002;169:2111–2119. [PubMed: 12165539]
31. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, Frees K, Watt JL, Schwartz DA. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000;25:187–191. [PubMed: 10835634]
32. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998;282:2085–2088. [PubMed: 9851930]
33. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat Med 2009;15:410–416. [PubMed: 19330007]
34. Koay MA, Gao X, Washington MK, Parman KS, Sadikot RT, Blackwell TS, Christman JW. Macrophages are necessary for maximal nuclear factor-kappa B activation in response to endotoxin. Am J Respir Cell Mol Biol 2002;26:572–578. [PubMed: 11970909]
35. Maus UA, Waelsch K, Kuziel WA, Delbeck T, Mack M, Blackwell TS, Christman JW, Schlondorff D, Seeger W, Lohmeyer J. Monocytes are potent facilitators of alveolar neutrophil emigration during lung inflammation: role of the CCL2-CCR2 axis. J Immunol 2003;170:3273–3278. [PubMed: 12626586]
36. Noulin N V, Quesniaux F, Schnyder-Candrian S, Schnyder B, Maillet I, Robert T, Vargaftig BB, Ryffel B, Couillin I. Both hemopoietic and resident cells are required for MyD88-dependent pulmonary inflammatory response to inhaled endotoxin. J Immunol 2005;175:6861–6869. [PubMed: 16272344]
37. Skerrett SJ, Liggitt HD, Hajjar AM, Ernst RK, Miller SI, Wilson CB. Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin. Am J Physiol Lung Cell Mol Physiol 2004;287:L143–152. [PubMed: 15047567]
38. McKinley L, Kim J, Bolgos GL, Siddiqui J, Remick DG. CXC chemokines modulate IgE secretion and pulmonary inflammation in a model of allergic asthma. Cytokine 2005;32:178–185. [PubMed: 16290175]
39. Monton C, Torres A. Lung inflammatory response in pneumonia. Monaldi Arch Chest Dis 1998;53:56–63. [PubMed: 9632909]
40. Hashimoto S, Pittet JF, Hong K, Folkesson H, Bagby G, Kobzik L, Frevert C, Watanabe K, Tsurufuji S, Wiener-Kronish J. Depletion of alveolar macrophages decreases neutrophil chemotaxis to Pseudomonas airspace infections. Am J Physiol 1996;270:L819–828. [PubMed: 8967517]
41. Broug-Holub E, Toews GB, van Iwaarden JF, Strieter RM, Kunkel SL, Paine R 3rd, Standiford TJ. Alveolar macrophages are required for protective pulmonary defenses in murine Klebsiella pneumonia: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. Infect Immun 1997;65:1139–1146. [PubMed: 9119443]
42. Hofer S, Rescigno M, Granucci F, Citterio S, Francolini M, Ricciardi-Castagnoli P. Differential activation of NF-kappa B subunits in dendritic cells in response to Gram-negative bacteria and to lipopolysaccharide. Microbes Infect 2001;3:259–265. [PubMed: 11334742]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

43. Iwasaki A. Mucosal dendritic cells. Annu Rev Immunol 2007;25:381–418. [PubMed: 17378762]
44. Angkasekwinai P, Park H, Wang YH, Wang YH, Chang SH, Corry DB, Liu YJ, Zhu Z, Dong C. Interleukin 25 promotes the initiation of proallergic type 2 responses. J Exp Med 2007;204:1509–1517. [PubMed: 17562814]
45. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM, Bazan JF, Kastelein RA. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity 2005;23:479–490. [PubMed: 16286016]
46. Stampfli MR, Wiley RE, Neigh GS, Gajewska BU, Lei XF, Snider DP, Xing Z, Jordana M. GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice. J Clin Invest 1998;102:1704–1714. [PubMed: 9802884]
47. Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB, Gyarmati D, Aye T, Campbell DJ, Ziegler SF. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. Nat Immunol 2005;6:1047–1053. [PubMed: 16142237]
48. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, Maisch S, Carr D, Gerlach F, Bufe A, Lauener RP, Schierl R, Renz H, Nowak D, von Mutius E. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med 2002;347:869–877. [PubMed: 12239255]
49. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, Thorne PS, Wills-Karp M, Gioannini TL, Weiss JP, Karp CL. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. Nature 2009;457:585–588. [PubMed: 19060881]
50. Wells CA, Chalk AM, Forrest A, Taylor D, Waddell N, Schroder K, Himes SR, Faulkner G, Lo S, Kasukawa T, Kawaji H, Kai C, Kawai J, Katayama S, Carninci P, Hayashizaki Y, Hume DA, Grimmond SM. Alternate transcription of the Toll-like receptor signaling cascade. Genome Biol 2006;7:R10. [PubMed: 16507160]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

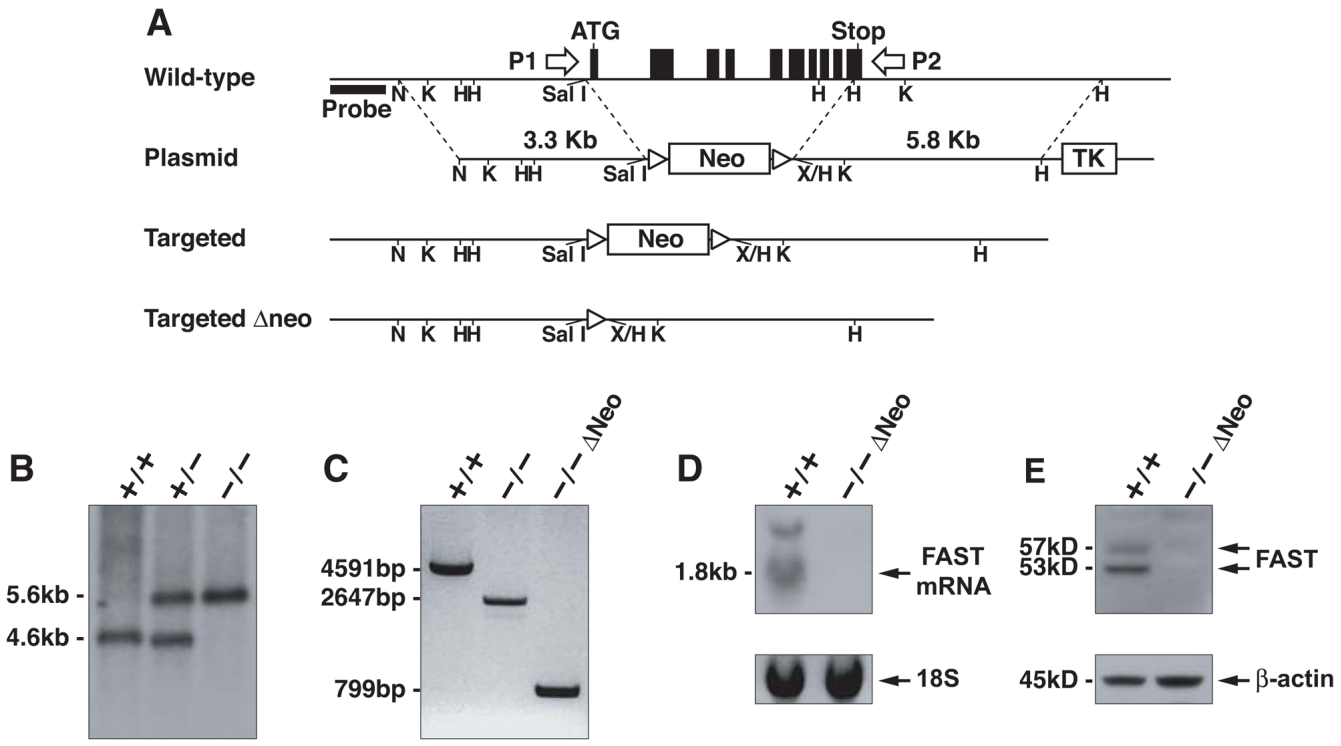

Figure 1. Generation of FAST$^{-/-}$ mice

(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 EIIa-Cre mice. Exons are indicated by black boxes. neo, neomycin resistance expression cassette; E, Ecl 136II; H, HindIII; K, KpnI; N, NotI; SI, SacI; X, XhoI. Open triangle: loxP site. (B) Southern blot analysis of DraI-digested genomic DNA from a 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 (MSG 419) distinguishes WT, FAST$^{-/-}$ neo+ (−/− neo+) and FAST$^{-/-}$ neo– (−/− Δneo) alleles. Primer sequences are provided in Supplemental Table I. (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 antibodies.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

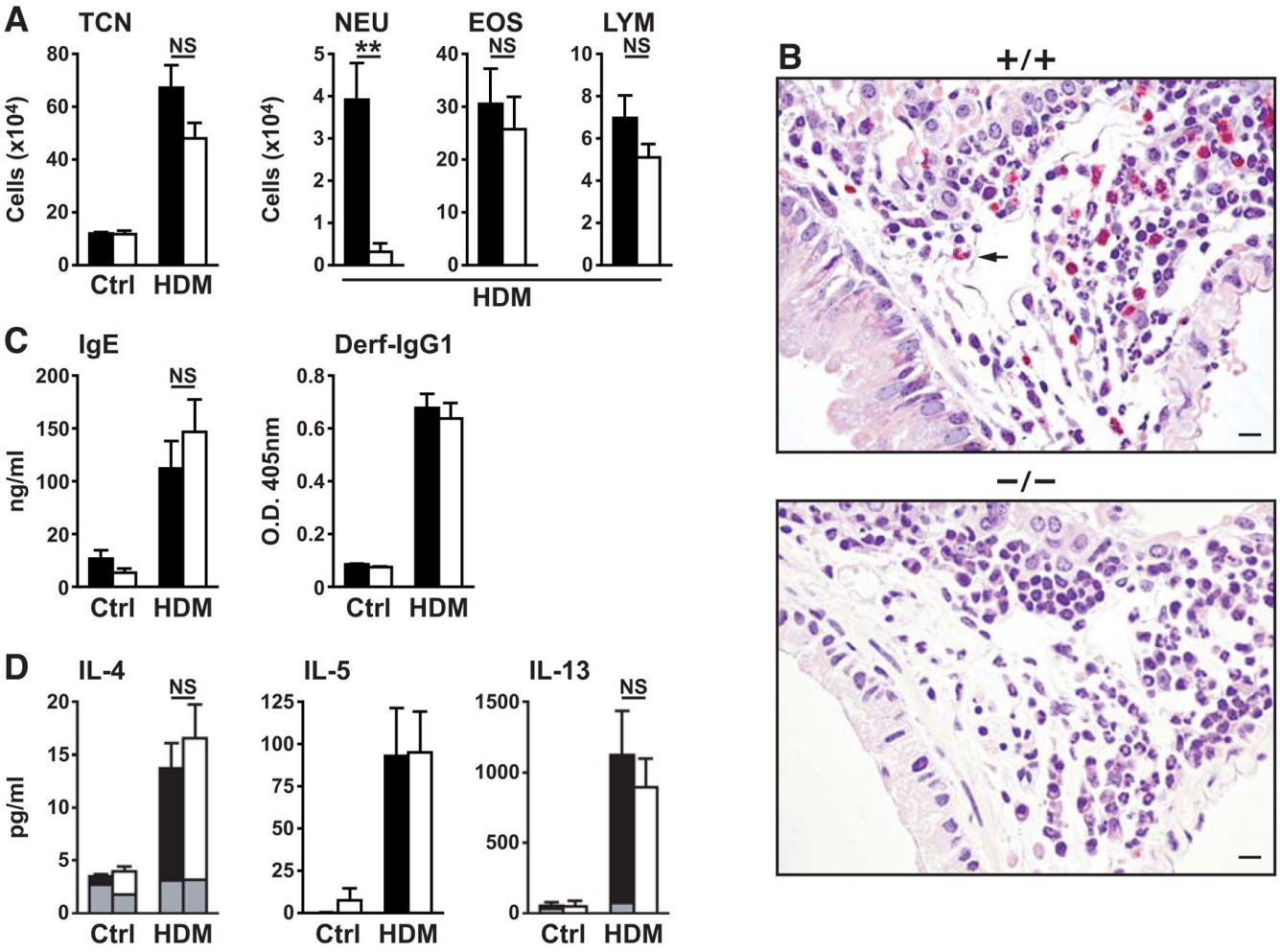

Figure 2. Lung inflammation in HDM-treated WT and FAST$^{-/-}$ mice

(A) Total and differential cell counts in BAL fluid. TCN, total cell number; NEU, neutrophils; EOS, eosinophils; LYM, lymphocytes. (B) Histological examination of inflammation around the bronchovascular bundles. Neutrophils (red-stained cells; arrow) were detected with chloroacetate esterase substrate. Scale bar= 10 μm. (C) Serum concentration of total IgE and HDM-specific IgG1 antibody 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 (grey bars) or presence of HDM (20 μg/mL). Control (Ctrl) WT and FAST$^{-/-}$ mice were challenged with sterile saline. Means ± SEM are shown (n=10). Black bars represent WT mice and white bars represent FAST$^{-/-}$ mice. $^{**}P < 0.01$.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

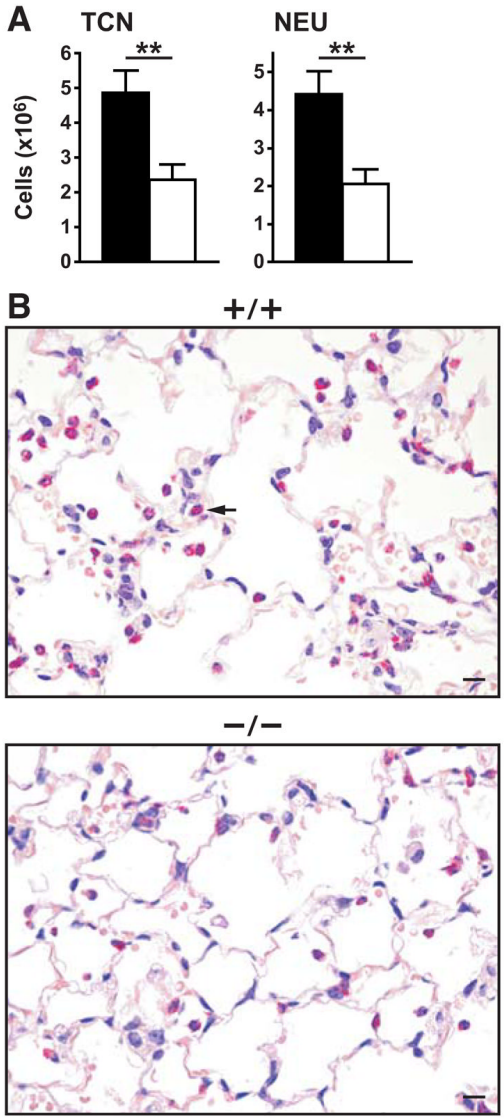

Figure 3. Lung inflammation in LPS-treated WT and FAST$^{-/-}$ mice

(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. Scale bar= 10 μm.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

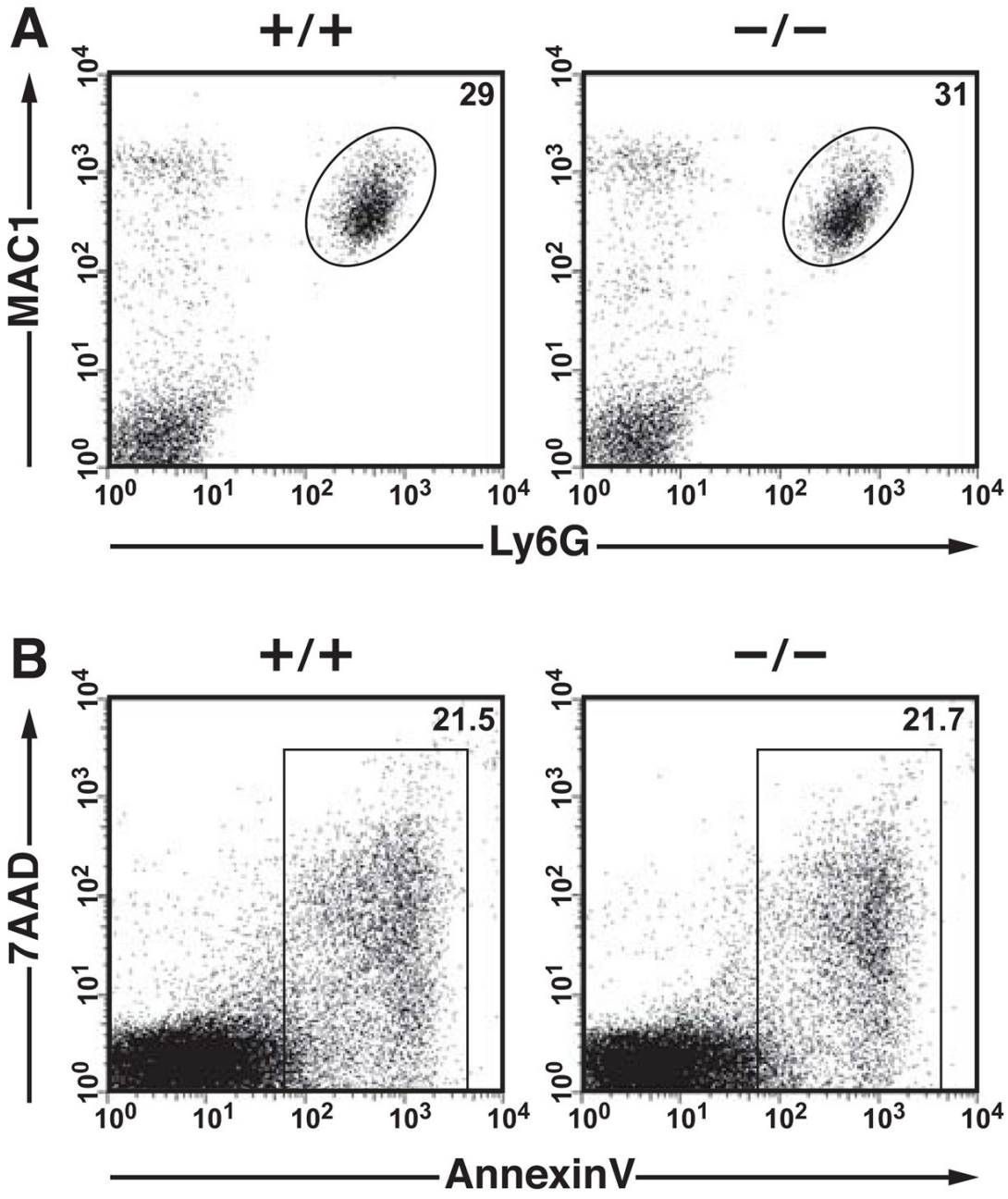

Figure 4.

(A) Representative FACS analysis of whole blood taken from LPS-treated mice. Neutrophils were stained with antibodies to Mac-1 and Ly6G. (B) FACS Analysis of Neutrophil Spontaneous Death. BM neutrophils were cultured in RPMI medium 1640 containing 10% FBS at a density of 2×10^6 cells/ml for 24 h. Apoptotic cells were detected by Annexin V-FITC and 7-AAD staining.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

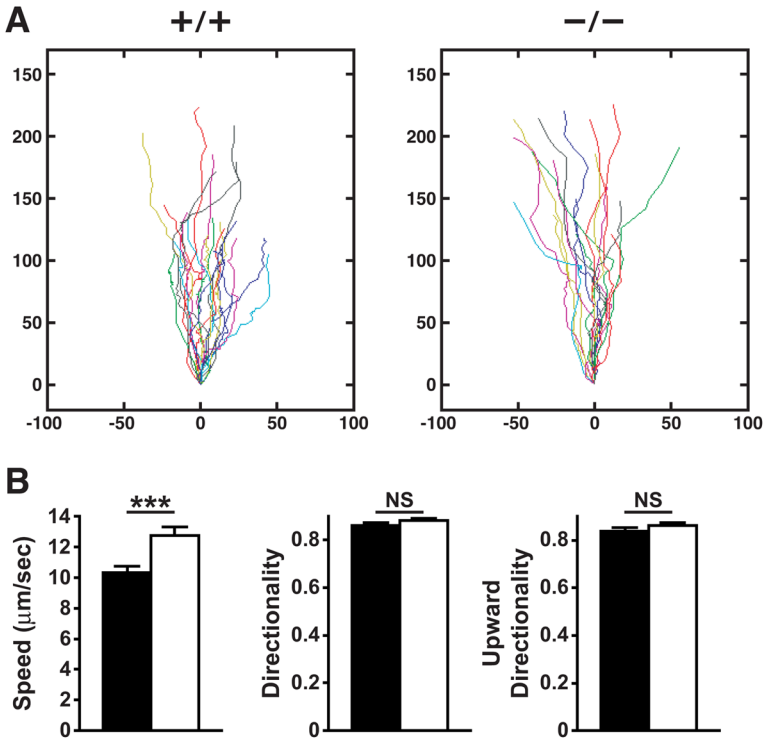

Figure 5. Chemotaxis in FAST$^{-/-}$ neutrophils

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 minutes with frames taken every 30 seconds. (A) Cell paths are shown starting at each origin. (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 3 different movie sequences. Black bars represent WT mice and white bars represent FAST$^{-/-}$ mice. ***$P < 0.001$.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

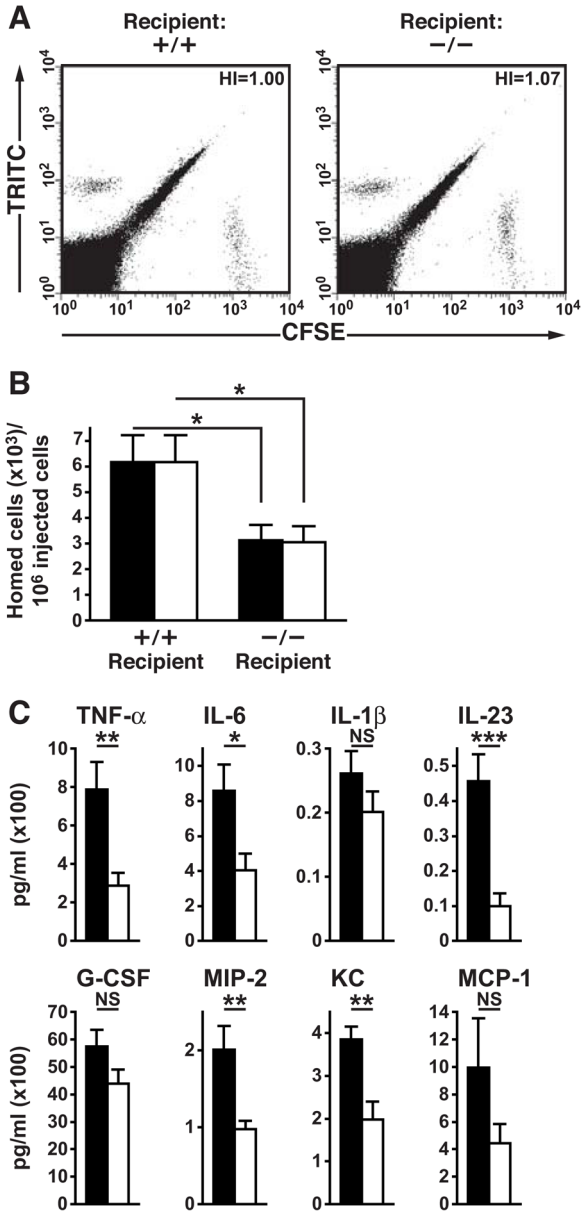

Figure 6. Recruitment of adoptively transferred neutrophils in LPS-treated WT and FAST$^{-/-}$

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 represents the ratio of CFSE+ to TRITC+ cells. HI, homing index. (B) Absolute number of adoptively transferred neutrophils recruited to the alveolar space. Data shown are means ± SEM of n=6 mice. *$P < 0.05$. (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. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

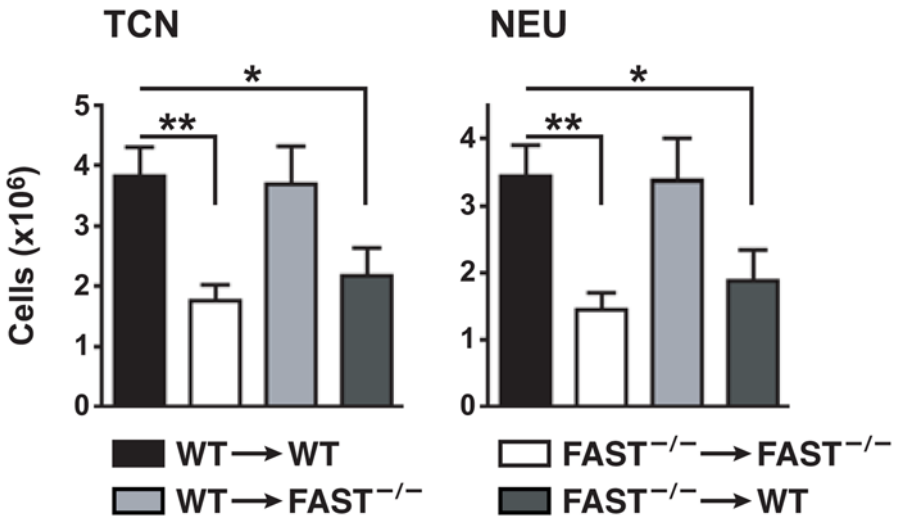

Figure 7. Lung inflammation in LPS-treated chimeric mice

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