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Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) Modulates Immune Responses to *Aspergillus fumigatus* During Fungal Asthma in Mice

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Abstract

Triggering receptor expressed on myeloid cells-1 (TREM-1) expression is increased during pulmonary fungal infection suggesting that this receptor might be involved in anti-fungal immune responses. To address the role of TREM-1 in a murine model of fungal allergic airway disease, *A. fumigatus*-sensitized CBA/J mice received by intra-tracheal injection a mixture of live *A. fumigatus* conidia and one of a control adenovirus vector (Ad70), an adenovirus containing a gene encoding for the extracellular domain of mouse TREM-1 and the F_c portion of human IgG (AdTREM-1Ig; a soluble inhibitor of TREM-1 function), or an adenovirus containing mouse DAP12 (AdDAP12; DAP12 is an intracellular adaptor protein required for TREM-1 signaling), and examined at various days after challenge. Whole lung TREM-1 levels peaked at day 3 whereas circulating TREM-1 levels peaked at day 30 in this fungal asthma model. AdTREM-1Ig-treated mice exhibited significantly higher airway hyperresponsiveness following methacholine challenge compared with Ad70- and AdDAP12-treated mice. Whole lung analysis of AdTREM-1Ig treated mice revealed markedly higher amounts of fungal material compared with the other groups. ELISA analysis of whole lung and bronchoalveolar lavage samples indicated that several pro-allergic cytokine and chemokines including CCL17 and CCL22 were significantly increased in the AdTREM-1Ig group compared with the other groups. Finally, Pam3Cys and soluble *Aspergillus* antigens induced TREM-1 transcript expression in macrophages in a TLR2 dependent manner. In conclusion, TREM-1 modulates the immune response directed against *A. fumigatus* during experimental fungal asthma.

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Keywords

Allergic Inflammation; Fungus; TREM-1

INTRODUCTION

Triggers for allergic pulmonary diseases are many and varied, but growing evidence suggests that exuberant fungal growth associated with dwellings, schools, and places of employment present a growing health hazard to individuals in all age groups (Kurup, 2000). Individuals with cutaneous and serologic evidence of *A. fumigatus* hypersensitivity typically exhibit the persistence of *A. fumigatus* conidia or fungal material incorporated into immune cells within airways, and this prolonged exposure to the fungus can cause airway hyperreactivity, peribronchial inflammation, and airway remodeling (Kauffman, 2000).

Allergic responses to *A. fumigatus* promote major complications frequently observed in individuals with cystic fibrosis (CF; up to 20% of patients) (Hartl, 2006), atopic individuals, and asthmatics (Holt, 1999). All allergic responses to *Aspergillus* appear to involve a number of immunologic abnormalities including: elevated IgE, enhanced Th2 cytokines such as IL-4, IL-5, and IL-13 (Skov, 1999); eosinophilic and T cell inflammation; and profound airway remodeling (Knutsen, 1994).

TREM-1 is an orphan receptor, which is highly expressed on the cell surface of neutrophils and monocytes and serves as an amplifier of toll-like receptor 2 (TLR2)- and TLR4-mediated immune responses (Bleharski, 2003). Due to the absence of signaling motif in its cytoplasmic tail, TREM-1 associates with the adapter protein DNAX-activation protein of 12kDa (DAP12) to propagate activation signals. TREM-1 actually shares DAP12 with a number of activating (i.e., MDL-1, PDC-TREM, etc) and inhibiting (i.e., TREM-2, NKp44, and Siglec-H) receptors. Membrane-anchored TREM-1 is found on neutrophils and CD14^{high} monocyte/macrophages (Bouchon, 2000, 2001), and is associated with a mature stage of differentiation (Gingras, 2002), and its expression is increased by the presence of LPS, bacteria or fungi (Bouchon, 2001). Ligation of TREM-1 on neutrophils *in vitro* induces secretion of pro-inflammatory cytokines and chemokines, immediate degranulation, respiratory burst and phagocytosis (Bleharski, 2003; Radsak, 2004). A soluble variant of TREM-1 (sTREM-1) also exists, and recent evidence suggests that it is generated through the proteolytic cleavage of membrane-bound TREM-1 via matrix metalloproteinase (MMP) activity rather than via the translation of a gene splice variant (Gomez-Pina, 2007).

Membrane-bound TREM-1 plays a critical regulatory role in immune responses during acute infection and inflammation (Dower, 2008; Ho, 2008). TREM-1 expression has also been reported in *Aspergillus*-containing granulomas, suggesting that this receptor might be involved in antifungal responses of the host (Bouchon, 2001). TREM-1 appears to be involved in inflammatory responses in multiple tissues, including the lung (Gibot, 2004). Soluble TREM-1 is present at high levels in serum and bronchoalveolar lavage (BAL) fluid from patients with infections (Gibot, 2004) and in plasma during sepsis in mice (Gibot, 2004), and appears to be released due to the activity of various metalloproteinases (Gomez-Pina, 2007).

The precise role of sTREM-1 during active infections is not known but exogenously administered sTREM-1 peptides have been shown to exert prominent anti-inflammatory roles in experimental sepsis (Gibot and Cravoisy, 2004). The downregulation of TREM-1 expression in monocytes from cystic fibrosis patients appears to partially contribute to the endotoxin tolerance state of these cells (del Fresno, 2007). Finally, we showed that TREM-1 and DAP-12 make important contributions to the innate immune response during invasive pulmonary aspergillosis in mice, and transgenic expression of a TREM-1 blocking protein via an adenovirus delivery system prevented fungal clearance and enhanced CCL17 expression (Carpenter, 2005).

Thus, the aim of the present study was to investigate whether *Aspergillus fumigatus* (*A. fumigatus*) altered TREM-1 levels during chronic fungal asthma and what effect the blockade of TREM-1 or the enhancement of the expression DAP12, had on the course of experimental allergic airway disease in mice. We observed that the tissue levels of TREM-1 were significantly enhanced in *A. fumigatus*-sensitized mice at day 3 after conidia, whereas soluble TREM-1 levels were highest at day 30 after conidia. The inhibition of TREM-1 activity via an adenovirus delivered TREM-1 blocker (i.e., AdTREM-1Ig) in a mouse model of fungal asthma markedly attenuated the clearance of *A. fumigatus* from the lung and aggravated physiologic and histologic features of allergic disease. AdTREM-1Ig-treated mice exhibited a significant increase in whole lung and BAL levels of pro-allergic cytokines and chemokines including CCL2, CCL17, and CCL22. Conversely, enhanced DAP12 expression markedly attenuated all features of allergic airway disease in mice. Together, these data indicate that TREM-1 and DAP12 are important for the clearance of *A. fumigatus* from the susceptible, allergic host.

METHODS

Mice

Female, CBA/J mice, C57BL/6 (TLR2^{+/+}), and TLR2^{-/-} (fully backcrossed on a C57BL/6 background) mice at 6–8 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME) and were maintained in a specific pathogen free University Laboratory of Animal Medicine facility at the University of Michigan. Prior approval for mouse usage was obtained from an University Committee on Use and Care of Animals at the University of Michigan.

Chronic Fungal Asthma Model

We have previously described a model of chronic allergic airway disease induced by *A. fumigatus* conidia (Hogaboam, 2000). CBA/J, TLR2^{+/+}, and TLR2^{-/-} mice were similarly sensitized to a commercially available preparation of soluble *A. fumigatus* antigens as previously described (Hogaboam, 2000). After completion of the sensitization protocol, all groups of mice (n = 5–10 mice/group) were anesthetized with ketamine and xylazine. The trachea of each CBA/J mouse was exposed in each anesthetized mouse using aseptic surgical techniques and 30 µl of sterile 0.1 % Tween 80 (i.e., vehicle) containing 5.0×10^6 *A. fumigatus* conidia (American Type Culture Collection strain 13073) thoroughly mixed with one of the following: saline alone, 2.0×10^8 PFU of a control adenovirus vector (Ad70), 2.0

10⁸ PFU of an adenovirus encoding for a fusion molecule containing the extracellular × domain of mouse TREM-1 and the F_c portion of human IgG1 (AdTREM-1 Ig), or 2.0 × 10⁸ PFU an adenovirus containing FLAG-DAP12 (AdDAP12). The construction of the latter two viral vectors is described in detail elsewhere (Nochi, 2003), and both constructs have been used *in vivo* to impair TREM-1 function through the elaboration of TREM-1 Ig (AdTREM-1 Ig) or enhance DAP12 (AdDAP12) expression, respectively (Nochi, 2003).

To examine the effects of the adenoviral vectors alone on the pulmonary immune system, other groups of non-sensitized CBA/J received one of the above vectors at 2.0 × 10⁸ PFU via intra-tracheal injection. *A. fumigatus*-sensitized TLR2^{+/+} and TLR2^{-/-} received 30 µl of sterile 0.1% Tween 80 (i.e., vehicle) containing 5.0 × 10⁶ *A. fumigatus* conidia alone by intratracheal injection as described above.

Assessment of Mouse Airway Physiology

Immediately prior to and at various days after the intratracheal *A. fumigatus* conidia ± adenovirus vector challenge in CBA/J mice, bronchial hyperresponsiveness was assessed in a BuxcoTM plethysmograph (Buxco, Troy, NY). After the assessment of airway hyperresponsiveness, approximately 500 µl of blood was removed from each mouse and centrifuged at 900 × *g* for 10 min to yield serum. BAL was then performed using 1 ml of filter-sterilized normal saline. Finally, whole lungs were dissected from each mouse and snap frozen in liquid N₂ or fixed in 10% formalin for histological analysis.

RNA Isolation and TAQMAN Analysis

Total RNA was isolated from snap-frozen mouse lung at various times after *A. fumigatus* conidia ± adenoviral vector challenge using Trizol reagent (Invitrogen/Life Technologies, Carlsbad, CA). Total RNA was also isolated from cultured bone marrow derived macrophages using Trizol reagent. Purified RNA from whole lung and macrophage samples was treated with DNase and reverse transcribed into cDNA using TAQMAN Reverse Transcription Reagents (Foster City, CA). Pre-developed TAQMAN Gene Expression Assays were used to quantify mouse MMP, DAP12, and TREM-1 transcripts as per the manufacturer's (Applied Biosystems) instructions.

Whole lung MMP transcript levels were normalized to GAPDH transcript level and then expressed as relative transcript expression. Whole lung DAP12 transcript levels were also normalized to GAPDH levels and expressed as fold changes in expression, which was calculated via the comparison of gene expression in naïve whole lung samples (assigned a value of 1) to that in the fungal asthma lung samples. Fold changes in TREM-1 expression in macrophages were similarly calculated. The amount of fungal material in paraffin wax embedded tissue sections, was determined using a method described and validated previously by Paterson and colleagues (Paterson, 2003, 2006). Briefly, a TaKaRa DEXPAT kit (TaKaRa Biomedicals, Shiga, Japan) with lyticase (Sigma, St Louis, USA) was used to extract DNA from the histological tissues sections and quantitative PCR analysis was then used to amplify *Aspergillus* 18S.

ELISA Analysis

Murine TREM-1, CCL2, IL-12p70, IL-13, CCL3, CCL17, CCL22, IL-10, CCL1, TNF- α , CCL5, and CCL6 were determined in 50- μ l samples of whole lung homogenates, BAL, and/or serum at various times prior to and after *A. fumigatus* conidia and $\pm$ adenoviral vector challenge using a standardized sandwich ELISA technique previously described in detail (Evanoff, 1992). Cell-free tissue culture supernatant samples were analyzed in a similar manner. The TREM-1, cytokine, and chemokine levels in each whole lung sample were normalized to total protein levels measured using a Bradford assay, unless otherwise indicated.

Immunocytochemical Analysis of BAL Cells for TREM-1 Expression

BAL samples at various days after the conidia $\pm$ adenovirus inoculation in CBA/J mice were cytospun onto glass slides and the cells were subjected to routine immune immunocytochemical analysis as described by our laboratory in detail elsewhere (Jakubick, 2004). A goat anti-mouse TREM-1 antibody from R&D Systems (Minneapolis, MN) was used for the detection of TREM-1 in these samples.

Serum Immunoglobulin Analysis

Serum levels of IgE and IgG2a at days 3 and 7 after conidia in non-sensitized and *A. fumigatus*-sensitized groups were analyzed using complementary capture and detection antibody pairs for IgE and IgG2a (PharMingen, San Diego, CA). Immunoglobulin ELISAs were performed according to the manufacturer's directions.

Whole Lung Histologic Analysis

Whole lungs from *A. fumigatus*-sensitized mice at days 7 and 28 after *A. fumigatus* conidia $\pm$ adenovirus challenge were fully inflated with 10% formalin, dissected, and placed in fresh 10% formalin for 24 h. Routine histological techniques were used to paraffin-embed the entire lung, and 5- μ m sections of whole lung were stained with one of the following: hematoxylin/eosin, periodic acid-Schiff reagent (PAS), Masson trichrome, or Gomori methanamine silver (GMS).

Bone Marrow Macrophage Isolation and Studies

Macrophages were cultured and activated as previously described in detail (Joshi, 2008). At day 3 after conidia challenge in *A. fumigatus*-sensitized TLR2^{+/+} and TLR2^{-/-} mice, bone marrow was derived by flushing femur and tibia bones with cold RPMI 1640 and the recovered bone marrow progenitor cells were cultured in L929 cell conditioned medium. Bone marrow was also removed from naïve TLR2^{+/+} mice. On day 7 after the initiation of the bone marrow culture, purified CD11b-positive bone marrow-derived macrophages were transferred to 24-well plates at a cell density of 2×10^5 cells/well, and 0.5 ml of RPMI 1640 containing 10% FBS, 100U/ml penicillin and 100 mg/ml streptomycin was added to each well.

One of the following was added to triplicate tissue culture plate wells containing bone marrow derived macrophages: Pam3cys (2.5 μ g/ml), Poly I:C (50 μ g/ml), LPS (10 μ g/ml),

CpG (50 µg/ml), CCL17 (10 ng/ml), gliotoxin (1 or 10 ng/ml; Sigma), or *Aspergillus* antigens (100 ng/ml or 1 µg/ml; Greer Laboratories). At 24 h, cell free supernatants were removed for ELISA analysis of TREM-1 levels or isolation of RNA for analysis of TREM-1 transcript levels. In separate experiments, macrophages were cultured with an anti-TLR2 monoclonal antibody (R&D Systems) or a TREM-1 blocker (recombinant mouse TREM-1/Fc chimeric protein; 1 µg/ml; R&D Systems). In cultures treated with the anti-TLR2 antibody, TREM-1 transcript levels were analyzed by quantitative PCR. Cell-free supernatants were subjected to ELISA analysis of IL-10 levels in cultures treated with the TREM-1 blocker.

Statistical Analysis

All results were expressed as mean ± standard error of the mean (SEM). A Student's T test or analysis of variance (ANOVA) and a Student-Newman-Keuls Multiple Comparison test were used to determine statistical significance between the control and treatment groups of mice at various times after the conidia challenge; $P < 0.05$ was considered statistically significant.

RESULTS

Increased sTREM-1 and Decreased Whole Lung Levels of TREM-1 During the Course of Experimental Fungus-Induced Allergic Airway Disease

We first examined the relative changes in TREM-1 during the course of experimental fungal allergic airway disease in wildtype mice. An analysis of whole lung, BAL, and serum samples from *A. fumigatus* sensitized CBA/J mice at various times after conidia challenge revealed that TREM-1 levels were dynamically altered over the course of experimental fungal asthma (Figure 1). Both BAL and serum levels appeared to track together as sTREM-1 levels were highest in both compartments at day 30 after conidia challenge. Whereas serum levels of sTREM-1 were stable over the first week after conidia challenge, BAL levels of sTREM-1 progressively increased with time. In contrast, whole lung levels of TREM-1 peaked at day 3 after conidia and returned to baseline (i.e., day 0) levels at day 30 after conidia (Figure 1). Thus, altered TREM-1 levels were apparent in whole lung, BAL, and serum during the course of experimental fungal asthma in mice.

MMP-9 Levels are Increased in a Time-Dependent Manner During Fungal Asthma

Given that MMP activity has been shown to be responsible for the shedding of the TREM-1 ectodomain (i.e., sTREM-1) through proteolytic mechanisms (Gomez-Pina, 2007), whole lung MMP transcript expression was examined during the course of this fungal asthma model. Using a quantitative TAQMAN approach to examine multiple MMP transcript levels in whole lung samples from *A. fumigatus*-sensitized C57BL/6 mice at days 15 and 30 after conidia, it was observed that MMP-9 was markedly increased at day 30 (Figure 2). Other MMP transcripts appeared to be altered to a lesser extent during chronic fungal asthma. These data suggested that the increase in sTREM-1 levels in both serum and BAL might be a consequence of increased MMP-9 expression and activity in the lungs of mice with *A. fumigatus*-induced allergic airway disease.

Adenovirus-mediated Gene Delivery of TREM-1 Ig and DAP12 Into the Lungs of Non-sensitized Mice and *A. fumigatus*-sensitized Mice with Allergic Airway Disease

We next addressed the effect of a TREM-1 blocker (i.e., TREM-1Ig) and the adaptor protein DAP12 during fungal asthma. Although sTREM-1 does not inhibit the function of membrane associated TREM-1, the TREM-1Ig construct used in the present study has been shown to block cell associated TREM-1 function (Nochi, 2003). In the present study, cells within the alveolar space of all groups of adenovirus and conidia-challenged mice were retrieved via BAL at day 3 after intratracheal injection, and adhered to glass slides via a cytopsin procedure. Immunostaining for TREM-1 revealed that granulocytes and mononuclear cells were positive for this protein in BAL samples from the Ad70 and AdDAP12 groups (Figures 3A, 3B, respectively), and TREM-1 staining appeared to be similar in both groups.

Immunoreactive TREM-1 levels were greatest in cells recovered from the BAL of the AdTREM-1-treated group at day 3, but the majority of this staining is TREM-1Ig rather than membrane-associated TREM-1 (Figure 3D). The antibody used in the immunostaining and ELISA procedures did not discriminate between native soluble TREM-1 and TREM-1 Ig, but we confirmed that the increased staining and increased protein expression detected by ELSIA was due to TREM-1 Ig and not native TREM-1 via immunodetection of the Ig F_C portion of the transgene (data not shown).

ELISA analysis of whole lung tissue confirmed that significantly greater amounts of TREM-1 protein were present in non-sensitized and *A. fumigatus*-sensitized mice due to the introduction of the AdTREM-1 Ig viral vector compared with all other viral, naïve and saline groups (Figure 3E). Whole lung levels of TREM-1 were significantly lower in Ad70-treated non-sensitized mice compared with the saline group but Ad70 treatment did not change whole lung TREM-1 levels in mice with fungal asthma. Immunoreactive levels of TREM-1 were also detected in BAL fluid of all groups of non-sensitized mice and the greatest levels of TREM-1 were observed in mice that received the AdTREM-1 Ig (Figure 3E). Prior sensitization to *A. fumigatus* antigens and conidia challenge substantially increased the quantity of TREM-1 in the BAL samples from all groups (Figure 3E). However, the greatest levels of immunoreactive TREM-1 were present in the BAL samples from the *A. fumigatus*-sensitized group that received AdTREM-1 Ig.

The presence of DAP12 in whole lung samples from the various groups of mice at day 3 after intratracheal challenge was measured using a real-time quantitative PCR technique (Figure 3F). DAP12 mRNA was increased approximately 3-fold in the *A. fumigatus*-sensitized and conidia-challenged control receiving either saline or Ad70 groups compared with naïve mouse lung suggesting that this gene is induced during sensitization to *A. fumigatus* antigens. In the AdDAP12 viral vector group, the levels of DAP12 mRNA expression were increased approximately 6-fold higher than those in naïve mice (Figure 3F). Finally, in the AdTREM-1 Ig viral vector group, DAP12 mRNA levels were increased approximately 5-fold above those in naïve mice. The increase in DAP12 transcript expression in this last group of mice might reflect a compensatory response to the presence of TREM-1 Ig. Thus, the transgene delivery of either TREM-1 Ig or DAP12 effectively

increased the expression of these gene products in the lungs of mice in the absence and presence of fungal allergy and allergic airway disease.

TREM-1 Ig Exacerbates Methacholine-induced Airway Hyperresponsiveness and IgE Levels in Mice with Chronic Fungal Allergic Airway Disease

Seven days after the intratracheal challenge with *A. fumigatus* conidia $\pm$ adenovirus, airway hyperresponsiveness was assessed via whole body plethysmography in anesthetized and ventilated mice. As shown in Figure 4A, the sequential intravenous introduction of 210 and 420 $\mu\text{g/kg}$ of methacholine promoted airway hyperresponsiveness in all groups of mice at days 3, 7, and 14 after intratracheal challenge. At day 3, significantly enhanced airway hyperresponsiveness in the AdTREM-1 Ig group (compared with the Ad70 group) was apparent following their challenge with the higher dose of methacholine but not with the lower dose of methacholine (Figure 4A).

At day 7, it was apparent that the instillation of the AdDAP12 viral vector altered the lung environment such that significantly lower airway hyperresponsiveness was provoked by in a dose-dependent manner by methacholine in this group compared with the Ad70 group. This difference in lower and higher dose methacholine-evoked airway hyperresponsiveness was again apparent in the AdDAP12-treated group at day 14 after the intratracheal challenge with viral vector and conidia (Figure 4A).

Airway hyperresponsiveness was measured in the saline and adenovirus groups at days 21 and 28 after conidia challenge and no statistically significant differences were observed between the groups at these time points (data not shown). Thus, compared with the Ad70 control group, these data showed that the presence of the DAP12 transgene in mice with fungal allergic lung disease significantly reduced whereas the presence of AdTREM-1Ig significantly enhanced airway hyperresponsiveness provoked by an intravenous methacholine challenge.

Another major feature of this experimental allergic airway model is the increased presence of immunoglobulins such as IgE and IgG2A (Hogaboam, 2000). Total IgE and IgG2A were measured in serum from groups of *A. fumigatus*-sensitized mice at day 3 (Figure 4B, top two panels) and non-sensitized and *A. fumigatus*-sensitized mice at day 7 (Figure 4B, bottom two panels) after intratracheal challenge. At day 3 after conidia, serum levels of IgE and IgG2a in the AdTREM-1Ig and AdDAP12 groups did not differ from the Ad70 control group (Figure 4B, top two panels).

However, at day 7 after vector injection into non-sensitized mice, total serum IgE was significantly increased in the AdDAP12 and AdTREM-1 Ig groups compared with the Ad70 group. Total IgG2A was also significantly increased in the AdTREM-1 Ig group of non-sensitized mice compared with the Ad70 group (Figure 4B). In the *A. fumigatus*-sensitized groups, total IgE was significantly elevated in the AdDAP12 and AdTREM-1 Ig groups compared with the Ad70 group. The levels of IgG2A were significantly increased in the AdDAP12, but not the AdTREM-1Ig group, compared with the Ad70 group. Thus, divergent effects on airway hyperresponsiveness and circulating Ig levels following AdTREM-1 Ig and AdDAP12 transgene expression were apparent in mice with fungal allergic airway disease.

AdTREM-1 Ig Exacerbates Goblet Cell Metaplasia, Peribronchial Fibrosis, Peribronchial Inflammation, and Fungal Retention in Mice with Chronic Fungal Allergic Airway disease

Fungal allergic airway disease is characterized by the metaplastic response of goblet cells in the airways and a peribronchial fibrotic response (Hogaboam, 2000). At day 7 after Ad70 and conidia challenge in *A. fumigatus*-sensitized mice, goblet cells were apparent in the airways of PAS-stained whole lung sections (Figure 5A). Although goblet cells were rarely detected in the airways of AdDAP12-challenged mice with chronic fungal lung disease (Figure 5B), a profound increase in goblet cells was apparent in mice that received AdTREM-1 Ig (Figure 5C). The presence of collagen around airways at day 28 after viral vector and conidia challenge in *A. fumigatus*-sensitized mice was highlighted via Masson trichrome staining as shown in Figure 5D (Ad70 group).

The AdDAP12 treatment (Figure 5E) did not diminish the degree of trichrome staining compared with the Ad70 group but trichrome staining was much more intense around the airways of AdTREM-1 Ig-treated group. Thus, blockade of TREM-1 function during the challenge of *A. fumigatus*-sensitized mice with *A. fumigatus* conidia markedly enhanced the amount of airway remodelling characterized by mucus cell metaplasia and peribronchial collagen deposition.

Our previous studies have revealed that the persistence of GMS-stained fungal material directly correlates with the severity of allergic airway disease. Specifically, the persistence of fungal material correlates with the severity of the peribronchial inflammatory response. In the present study, it was apparent that the presence of Ad70 and conidia in the lungs of *A. fumigatus*-sensitized mice provoked a dramatic inflammatory response at day 7 after intratracheal challenge (Figure 6A).

The lungs of these mice also contained several cells that stained positive for the presence of fungal material (Figure 6D). The AdDAP12 treatment dramatically decreased both the amount of peribronchial inflammation (Figure 6B) and GMS-positive cells (Figure 6E). Conversely, AdTREM-1 Ig-treated mice exhibited profound pulmonary inflammation with an extensive inflammatory cell infiltrate observed around the airways and throughout the lung parenchyma (Figure 6C). Coincident with this enhanced inflammatory response was the substantial presence of fungal material within the lung (Figure 6F).

Further confirmation that blockade of TREM-1 affected the presence of fungal material in the lungs of mice with fungal asthma was obtained using a quantitative molecular method for the identification of *Aspergillus* in paraffin wax embedded whole lung samples (Paterson, 2003). As shown in Figure 6G, the molecular signature of *Aspergillus* conidia was present in Ad70, AdTREM-1 Ig, and the AdDAP-12 groups at days 14, 21, and 28 after conidia challenge.

However, the expression of fungal conidia transcripts was consistently higher in the AdTREM-1 Ig-treated group compared with the Ad70 groups. The greatest fungal transcript levels were observed at the day 14 time point in the AdTREM-1 Ig group. Thus, TREM-1 blockade markedly inflamed the lungs of *A. fumigatus*-sensitized mice challenged with *A. fumigatus* conidia. Nevertheless, this inflammatory response appeared to be ineffective in

the clearance of fungal material from the lungs of these mice as greater amounts of fungal material were present in the lungs of these mice.

Whole Lung Cytokine and Chemokine Levels Were Significantly Enhanced Following Blockade of TREM-1 During Experimental Fungal Asthma

Considerable evidence points to the important role of cytokines and chemokines in the initiation and maintenance of fungal allergic responses in the lung (Schuh, 2003; Hartl, 2006). At day 7 after intratracheal challenge, whole lung levels of CCL2 were significantly increased in the AdTREM-1 Ig compared with the other viral vector groups examined at this time (Figure 7A).

IL-12 levels were significantly increased in both the AdDAP12 and AdTREM-1 Ig groups compared with the Ad70 control group at day 14 after conidia (Figure 7B). Also at this time, we observed that IL-13 was significantly lower in the AdTREM-1 Ig group compared with the Ad70 group, whereas CCL22 was significantly higher when these two groups were compared (Figure 7B). CCL2, CCL3, and CCL17 levels were significantly higher in the AdTREM-1 Ig group compared with the AdDAP-12 group (Figure 7B).

At day 21 after intratracheal challenge, IL-10 and CCL2 were significantly decreased in the AdTREM-1 Ig-treated group compared with the saline group (Fig. 7C). Whole lung CCL1 levels were significantly elevated in the AdDAP12 group compared with the Ad70 control group and the saline group at this time (Figure 7C). TNF- α and IL-13 were significantly decreased in whole lung samples from the AdTREM-1 Ig group compared with the other three groups at day 21 after intratracheal challenge (Figure 7D). Whole lung IL-12 levels in all three adenovirus groups were significantly higher than those levels in the saline group (Figure 7D).

It is of note that the saline group was included in our analysis of whole lung samples at day 21 after challenge because of the above noted differences between this group and the adenoviral treated groups. These differences were unique to this timepoint. Finally, major differences in whole lung levels of cytokine and chemokines were still apparent at day 28 after intratracheal challenge with viral vector and conidia (Figure 7E).

Specifically, whole lung levels of TNF α , IL-12, CCL2, CCL3, CCL5, CCL17 and CCL22 were all significantly elevated in the AdTREM-1 Ig group compared with the Ad70 group (Figure 7E). Whole lung levels of CCL2 and CCL17 were both significantly decreased in the lung tissue from AdDAP12-challenged mice compared with Ad70-challenged mice (Figure 7E). Together, several proallergic chemokines including CCL2, CCL3, CCL5, CCL17, and CCL22 were significantly elevated in whole lung samples from the AdTREM-1 Ig group relative to the Ad70 group during the course of allergic airway disease perhaps explaining the exacerbated allergic airway disease in this group.

BAL Levels of Cytokine and Chemokine Levels Were Markedly Altered by the Presence of AdTREM-1 Ig

Because of the expression of TREM-1 Ig in cells from the alveolar compartment, we next examined the presence of pro-allergic cytokines and chemokines in the BAL of these groups

of mice at days 3, 7, 14, 21, and 28 after intratracheal challenge with saline or viral vectors and conidia. Although IL-10 levels were significantly lower in the AdTREM-1 Ig group compared with the Ad70 group at day 14 after intratracheal challenge, both IL-12 and CCL22 were significantly increased in the AdTREM-1 Ig and AdDAP12 groups compared with the Ad70 control group (Figure 8C).

At day 21 after intratracheal challenge, CCL22 was significantly increased in BAL samples from the AdTREM-1 Ig group compared with the other control and viral vector groups (Figure 8D). Also at this time, BAL levels of IL-13 were significantly lower whereas BAL IL-12 levels were significantly increased in both AdDAP12 and AdTREM-1 groups compared with the Ad70 group (Figure 8D). Finally, IL-12 was significantly raised in the BAL from the AdDAP12 and AdTREM-1 Ig groups compared with the Ad70 group at day 28 after intratracheal challenge with viral vector and conidia. Together, these data highlighted that the presence of AdTREM-1 Ig increased several pro-allergic mediators including CCL6 (Hogaboam, 1999), CCL17, and CCL22 (Schuh, 2002) compared with the Ad70 control group.

TREM-1 Expression is TLR2-dependent and is Induced by Soluble Aspergillus Antigens

We have previously examined the role of TLR2 in this fungal asthma model and observed that TLR2^{-/-} mice develop a persistent and more severe form of the disease compared with TLR2^{+/+} mice because of the retention of fungal material in the lung (Buckland, 2008). Given the similar defects in the anti-fungal responses in allergic TLR2^{-/-} mice and the wild-type allergic mice in which TREM-1 was targeted in the present study, we next determined what role TLR activation had upon TREM-1 expression in macrophages.

In cultures of bone marrow derived macrophages from *A. fumigatus*-sensitized and challenged TLR2^{+/+} mice at day 3 after conidia, a quantitative TAQMAN PCR analysis revealed that Pam3Cys (a TLR2 ligand) was a potent inducer of TREM-1 transcript expression approximately 5-fold above levels measured in naïve macrophages (Figure 9A). Poly I:C (a TLR3 ligand) and CpG (a TLR9 ligand) had no effect on the induction of TREM-1, whereas LPS (a TLR4 ligand) induced TREM-1 approximately 1.5 fold (Figure 9A).

Conversely, in cultures of bone marrow derived macrophages from *A. fumigatus*-sensitized and challenged TLR2^{-/-} mice at day 3 after conidia, Pam3Cys and LPS failed to induce TREM-1 expression (Figure 9A). Additional experiments addressed whether antigens from *A. fumigatus* induced the expression of TREM-1 in a TLR2-dependent manner, bone marrow macrophages from allergic TLR2^{+/+} mice at day 3 after conidia challenge were exposed to gliotoxin alone or a clinical isolate of *A. fumigatus* antigens (used to screen patients for hypersensitivity to this fungus) for 24 h and then analyzed using quantitative PCR for TREM-1 transcript expression. Gliotoxin is an antibiotic made by *A. fumigatus*, but this toxin can have direct effects on immune cells. We tested whether this was perhaps the component in the clinical isolate of *Aspergillus* antigens that induced TREM-1 expression.

As shown in Figure 9B, the presence of gliotoxin at 1 and 10 ng/ml did not alter TREM-1 transcript expression but the presence of *A. fumigatus* antigens dose-dependently increased

TREM-1 transcript expression. However, the inclusion of a monoclonal anti-TLR2 antibody markedly inhibited TREM-1 transcript expression in other macrophage cultures (Figure 9B). Thus, activation through TLR2 provides key activation events in macrophages leading to the induction of TREM-1 expression and *A. fumigatus* antigens drive the expression of TREM-1 in a TLR2-dependent manner.

TLR2 Activation and CCL17 Promote the Release of sTREM-1 from Macrophages and IL-10 Generation by Macrophages is TREM-1-Dependent

Although the release of TREM-1 might be an important regulatory mechanism during inflammatory responses, we hypothesized that the release of TREM-1 from the surface of immune cells might be one mechanism through which this fungus persists in the lungs of allergic mice. To test this hypothesis, we examined bone marrow-derived macrophages from allergic mice at day 3 after conidia challenge for their generation of sTREM-1 following exposure to Pam3Cys, hypomethylated DNA (CpG) or CCL17 for 24 h. The addition of Pam3Cys or CCL17 to cultures of allergic macrophages significantly increased sTREM-1 levels in these cultures (Figure 10A). Together, these data suggested that TLR2 or CCR4 activation lead to the release of TREM-1 from macrophages.

IL-10 is a key regulatory cytokine during innate and adaptive immune responses to *A. fumigatus*. Its absence during in immunocompromised hosts enhances their ability to clear this fungal pathogen (Clemons, 2000), whereas IL-10 is critically important in protecting the host from the deleterious effects of allergen-induced pulmonary inflammation (Grunig, 1997). To examine the role of TREM-1 in cytokine generation by *A. fumigatus*-activated macrophages, we examined whether IL-10 was induced by *A. fumigatus*-antigens in a TREM-1-dependent manner. The data from the analysis of IL-10 levels in cultures of *A. fumigatus*-activated macrophages grown from naïve mice is shown in Figure 10B.

The lower dose of *A. fumigatus* antigens induced IL-10 synthesis by macrophages and this effect was dependent upon the activation of TREM-1 (Figure 10B). Thus, a component present in the crude extract of antigen *Aspergillus* antigens promoted IL-10 generation by bone marrow macrophages, which was TREM-1 dependent.

DISCUSSION

In the present study we explored the role of TREM-1 and DAP12, an adaptor protein that facilitates signalling through TREM-1 and other immunoreceptors containing the tyrosine-based activation motif (ITAM), in a well-established murine model of chronic fungal asthma. TREM-1 and DAP12 contributed to the innate immune response needed for effective clearance of fungal conidia from the lungs of *A. fumigatus*-sensitized mice.

Accordingly, the blockade of cell associated TREM-1 activity with a soluble TREM-1 Ig blocking chimeric protein introduced via an adenoviral vector resulted in the persistence of fungal material in the lung, which was associated with enhanced circulating IgE, profound inflammation in the lung, increased AHR, and enhanced remodelling of the airways of mice with experimental fungus-induced allergic airway disease. Conversely, *A. fumigatus*-sensitized mice subjected to adenovirus-induced DAP12 expression during conidia challenge

exhibited significantly less IgE, AHR, and airway remodelling. A number of cytokines and chemokines were altered in mice receiving the TREM-1 Ig transgene but increased CCL17 and CCL22 was consistently observed in both whole lung and BAL samples from these mice at various times after viral vector and conidia challenge compared with the other groups.

In AdDAP12-treated mice, whole lung and BAL levels of CCL17 and CCL22 were generally significantly lower than levels of these chemokines in the AdTREM-1 Ig and Ad70 groups. In macrophages, TREM-1 expression was induced Pam3Cys or *Aspergillus* antigens in a TLR2-dependent manner, and the generation of IL-10 from macrophages exposed to *Aspergillus* antigens was dependent upon membrane-associated TREM-1. Together, these data indicate that the innate immune response mediated by TREM-1 was required for the modulation of the allergic airway response to *A. fumigatus* conidia in an experimental fungal asthma model.

Soluble TREM-1 levels have been extensively monitored in a number of acute inflammatory clinical conditions (Colonna and Facchetti, 2003), and it has been shown that the increase in soluble levels might be related to increased metalloproteinase activity typically associated with these conditions (Gomez-Pina, 2007). Although it is known that TREM-1 is involved in the amplification of TLR2 and TLR4 (Bouchon, 2001) and the NACHT-LRR (NLR) (Netea, 2006), it is not clear whether TREM-1 on the cell surface binds directly to pathogen associated molecular patterns (Klesney-Tait and Colonna, 2007).

An endogenous TREM-1 ligand has been identified on human platelets but the identity of this ligand is not presently clear (Haselmayer, 2007). In the present study, we observed that circulating TREM-1 levels were greatest in mice with fungal asthma at day 30 after conidia challenge, coinciding with markedly increased transcript levels of MMP-9 in whole lung. In contrast, the whole lung levels of membrane-associated TREM-1 peaked at day 3 after conidia challenge. Membrane-anchored TREM-1 protein was detected in granulocytes and mononuclear cells present in the BAL samples of Ad70 and AdDAP12-treated mice at day 3 after conidia, and AdTREM-1 Ig was present in these same cells in AdTREM-1 Ig-treated mice.

Interestingly, whole lung levels of TREM-1 did not differ between non-sensitized and *A. fumigatus*-sensitized mice at day 7 after conidia challenge. However, BAL levels of TREM-1 were approximately 25-fold higher in mice with fungal allergic pulmonary disease at day 7 after conidia compared with non-sensitized mice not challenged with conidia, suggesting that the primary cellular sources of TREM-1 were found on cells within the alveolar compartment. Further confirmation of the importance of alveolar cells in the generation of TREM-1 during fungal allergy responses was observed in the *A. fumigatus*-sensitized group that received Ad70.

In this group, sTREM-1 levels in the BAL of the Ad70-treated sensitized group were significantly higher than sTREM-1 levels in the saline-treated sensitized group. Given that we have shown that control adenovirus alone exacerbates fungal allergic airway disease in mice (Blease, 2001), it is possible that one mechanism for this exacerbation might be due to the release of sTREM-1 from immune cells in the alveolar compartment. Thus, given

prominent alterations in the localization of TREM-1 expression, it is possible that the progressive increase in sTREM-1 levels contributes to the chronicity of this model.

The AdTREM-1 Ig was constructed to contain the extracellular domain of mouse TREM-1 and the Fc portion of human IgG1 (Ad-TREM-1 Ig) whereas AdDAP12 contains a murine DAP12 gene (Nochi, 2003). The rationale behind the use of a human IgG1 rather than mouse IgG1 stems from the potent regulatory role of IgG alone in inflammatory responses; the use of human IgG1 eliminated these non-specific effects of mouse IgG1.

In a mouse model of hepatic granulomatous inflammation elicited by zymosan A, the transgene expression in the liver of soluble form of extracellular domain of TREM-1 inhibited zymosan A-induced granuloma formation whereas AdDAP12 had the opposite effect (Nochi, 2003). In the present study, we found that delivery of these adenoviral vectors to the lungs of mice induced the prominent expression of appropriate target gene, either TREM-1 Ig or DAP12. Immunohistochemical analysis of whole lung samples indicated that the major site of TREM-1 Ig expression in viral vector challenged allergic mice was the airway epithelium (data not shown), which is consistent with the fact that adenoviral vectors effectively target the respiratory epithelium (Gauldie, 1996).

We focused on the measurement of the transgenes over the first week after intratracheal challenge because the levels of transgene delivered by replication deficient adenoviruses are transient and typically wane after the first week (Gauldie, 1996). Nevertheless, long-term changes in airway inflammation and remodeling were observed most dramatically in those mice that received the AdTREM-1 Ig vector. Together, these data indicate that the early innate immune anti-fungal response mediated by TREM-1 and DAP12 have major roles in both the innate and adaptive immune responses to *A. fumigatus*, and disruption in their function leads to fungal asthma exacerbation.

Aspergillus fumigatus conidia enhance the expression of Ig in *A. fumigatus*-sensitized mice, which is detected in serum at various times after conidia challenge (Hogaboam, 2003). Although IgE (as a marker of hypersensitivity to *Aspergillus* (Kurup, 1999)) and IgG2a (as a marker of an immune shift from a Th2 to a Th1 cytokine phenotype during Aspergillosis (Kurup, 1997)) are routinely monitored in allergic aspergillosis, we (Blease, 2000, 2002; Schuh, 2002) and others (Corry, 1998) have confirmed that antibodies are dispensable during the development and maintenance of airway inflammation, airway hyperresponsiveness, and airway remodeling.

In the present study, the highest levels of circulating total IgE were observed in the AdTREM-1 Ig, whereas the lowest levels of circulating total IgE were observed in the AdDAP12 group at day 3 after conidia. However, both AdTREM-1 Ig and AdDAP12 increased the expression of total IgE, and Ad TREM-1 Ig increased the expression of total IgG2a in non-sensitized mice receiving the viral vector alone 7 days previously. This effect was not a feature of the Ad70 vector as this vector alone had no effect on either IgE or IgG2a levels.

It is not presently clear as to how these vectors are driving these Igs, however, analysis of BAL and whole lung samples from non-sensitized mice that received the adenovirus vectors

alone revealed that AdTREM-1 Ig-treated group had significantly higher levels of CCL2, CCL17, and CCL22 (CMH, unpublished findings). Thus, TREM-1 and DAP12 appear to have major roles in the regulation of circulating Ig levels in non-allergic and allergic mice, and this regulation might be related to their effects on chemokines with known B cell activating properties.

The presence of TREM-1 Ig enhanced the retention of fungal material in the lungs of *A. fumigatus*-sensitized mice and we observed increased levels of CCL17 and CCL22, another CCR4-specific ligand (Imai, 1998), in *A. fumigatus*-sensitized mice that received AdTREM-1 Ig and conidia. These two observations were mutually connected since we have previously observed that CCL17 impairs the pulmonary anti-fungal response during invasive Aspergillosis (Carpenter and Hogaboam, 2005). This impairment might be related, in part, to the potent effect CCL17 has on the activation status of macrophages, driving these cells toward M2 activation and away from an effective antifungal immune response.

Accordingly, our present finding revealed that CCL17 promoted the release of TREM-1 from the cell surface of M2 macrophages but this effect was not observed in cultures of untreated or CpG-treated macrophages. Although Pam3Cys also promoted the release of TREM-1 from macrophages, it promoted the expression of TREM-1 transcripts within these cells in a TLR2-dependent manner. Under similar conditions, CCL17 alone did not increase TREM-1 transcript levels in cultured macrophages (CMH, unpublished findings). Thus, the cytokine and chemokine findings in the present study are consistent with the putative role of these factors in the modulation of anti-fungal immune responses in mice.

How does the modulation of TREM-1 activation during experimental fungal asthma regulate the allergic response? The answer to this question is not fully apparent at present but the diminution in IL-10 generation observed in the *in vivo* and *in vitro* studies following TREM-1 blockade might provide a mechanism. IL-10 has potent suppressive and modulatory effects on innate immune responses directed towards *Aspergillus* as it has been shown to suppress the oxidative burst and antifungal activity of mononuclear cells toward *Aspergillus* but increase the phagocytic activity of these cells (Roilides, 1997).

IL-10 also has potent modulatory effects on the adaptive immune responses as it has been shown to inhibit Th2-type inflammatory responses during experimental allergic aspergillosis (Grunig, 1997; Montagnoli, 2006). During innate and adaptive immune responses against *Aspergillus*, the macrophage (Grunig, 1997) and the DC (Roilides, 1997) are likely major sources of this cytokine because their internalization of *A. fumigatus* conidia promotes IL-10 generation.

In the present study, *Aspergillus* antigen promoted the expression of IL-10 by bone marrow-derived macrophages in a TREM-1-dependent manner. These findings are the first to demonstrate that some component of *Aspergillus* antigen preparation activates macrophages in this manner. Thus, the inhibition of IL-10 generation as a consequence of preventing *Aspergillus* antigen-induced TREM-1 activation might have marked effects on both the innate and adaptive immune responses during experimental fungal asthma.

In conclusion, we observed that the innate and adaptive immune responses mediated by TREM-1 were critical for the clearance of *A. fumigatus* conidia from the lungs of *A. fumigatus*-sensitized mice. Without the cell-associated function of TREM-1, allergic mice exhibited an exacerbated form of lung disease characterized by increased airway inflammation, airway hyperresponsiveness, and airway remodeling.

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References

- Blease K, Mehrad B, Lukacs NW, Kunkel SL, Standiford TJ, Hogaboam CM. Antifungal and airway remodeling roles for murine monocyte chemoattractant protein-1/CCL2 during pulmonary exposure to *Aspergillus fumigatus* conidia. *J Immunol.* 2001; 166(3):1832–1842. [PubMed: 11160230]
- Blease K, Mehrad B, Standiford TJ, Lukacs NW, Kunkel SL, Chensue SW, Lu B, Gerard CJ, Hogaboam CM. Airway remodeling is absent in CCR1^{-/-} mice during chronic fungal allergic airway disease. *J Immunol.* 2000; 165(3):1564–1572. [PubMed: 10903765]
- Blease K, Schuh J, Jakubzick C, Lukacs NW, Kunkel SL, Joshi BH, Puri RK, Kaplan MH, Hogaboam CM. Stat6-deficient mice develop airway hyperresponsiveness and peribronchial fibrosis during chronic fungal asthma. *Am J Pathol.* 2002; 160:481–490. [PubMed: 11839568]
- Bleharski JR, Kiessler V, Buonsanti C, Sieling PA, Stenger S, Colonna M, Modlin RL. A role for triggering receptor expressed on myeloid cells-1 in host defense during the early-induced and adaptive phases of the immune response. *J Immunol.* 2003; 170(7):3812–3818. [PubMed: 12646648]
- Bouchon A, Dietrich J, Colonna M. Cutting edge: Inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol.* 2000; 164(10):4991–4995. [PubMed: 10799849]
- Bouchon A, Facchetti F, Weigand MA, Colonna M. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature.* 2001; 410(6832):1103–1107. [PubMed: 11323674]
- Buckland KF, O'Connor E, Murray LA, Hogaboam CM. Toll like receptor-2 modulates both innate and adaptive immune responses during chronic fungal asthma in mice. *Inflamm Res.* 2008; 57(8):379–387. [PubMed: 18787777]
- Carpenter KJ, Buckland KF, Xing Z, Hogaboam CM. Intrapulmonary, adenovirus-mediated overexpression of KARAP/DAP12 enhances fungal clearance during invasive aspergillosis. *Infect Immun.* 2005; 73(12):8402–8406. [PubMed: 16299339]
- Carpenter KJ, Hogaboam CM. Immunosuppressive effects of CCL17 on pulmonary antifungal responses during pulmonary invasive aspergillosis. *Infect Immun.* 2005; 73(11):7198–7207. [PubMed: 16239514]
- Clemons KV, Grunig G, Sobel RA, Mirels LF, Rennick DM, Stevens DA. Role of IL-10 in invasive aspergillosis: Increased resistance of IL-10 gene knockout mice to lethal systemic aspergillosis. *Clin Exp Immunol.* 2000; 122(2):186–191. [PubMed: 11091273]
- Colonna M, Facchetti F. TREM-1 (triggering receptor expressed on myeloid cells): A new player in acute inflammatory responses. *J Infect Dis.* 2003; 187(Suppl 2):S397–401. [PubMed: 12792857]
- Corry DB, Grunig G, Hadeiba H, Kurup VP, Warnock ML, Sheppard D, Rennick DM, Locksley RM. Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. *Mol Med.* 1998; 4(5):344–355. [PubMed: 9642684]
- del Fresno C, Soler-Rangel L, Soares-Schanoski A, Gomez-Pina V, Gonzalez-Leon MC, Gomez-Garcia L, Mendoza-Barbera E, Rodriguez-Rojas A, Garcia F, Fuentes-Prior P, Arnalich F, Lopez-Collazo E. Inflammatory responses associated with acute coronary syndrome up-regulate IRAK-M

- and induce endotoxin tolerance in circulating monocytes. *J Endotoxin Res.* 2007; 13(1):39–52. [PubMed: 17621545]
- Dower K, Ellis DK, Saraf K, Jelinsky SA, Lin LL. Innate immune responses to TREM-1 activation: Overlap, divergence, and positive and negative cross-talk with bacterial lipopolysaccharide. *J Immunol.* 2008; 180(5):3520–3534. [PubMed: 18292579]
- Evanoff H, Burdick MD, Moore SA, Kunkel SL, Strieter RM. A sensitive ELISA for the detection of human monocyte chemoattractant protein-1 (MCP-1). *Immunol Invest.* 1992; 21:39–49. [PubMed: 1548046]
- Gauldie J, Graham F, Xing Z, Braciak T, Foley R, Sime PJ. Adenovirusvector-mediated cytokine gene transfer to lung tissue. *Ann NY Acad Sci.* 1996; 796:235–244. [PubMed: 8906231]
- Gibot S, Cravoisy A. Soluble form of the triggering receptor expressed on myeloid cells-1 as a marker of microbial infection. *Clin Med Res.* 2004; 2(3):181–187. [PubMed: 15931355]
- Gibot S, Cravoisy A, Levy B, Bene MC, Faure G, Bollaert PE. Soluble triggering receptor expressed on myeloid cells and the diagnosis of pneumonia. *N Engl J Med.* 2004; 350(5):451–458. [PubMed: 14749453]
- Gibot S, Kolopp-Sarda MN, Bene MC, Cravoisy A, Levy B, Faure GC, Bollaert PE. Plasma level of a triggering receptor expressed on myeloid cells-1: its diagnostic accuracy in patients with suspected sepsis. *Ann Intern Med.* 2004; 141(1):9–15. [PubMed: 15238365]
- Gingras MC, Lapillonne H, Margolin JF. TREM-1, MDL-1, and DAP12 expression is associated with a mature stage of myeloid development. *Mol Immunol.* 2002; 38(11):817–824. [PubMed: 11922939]
- Gomez-Pina V, Soares-Schanoski A, Rodriguez-Rojas A, Del Fresno C, Garcia F, Vallejo-Cremades MT, Fernandez-Ruiz I, Arnalich F, Fuentes-Prior P, Lopez-Collazo E. Metalloproteinases shed TREM-1 ectodomain from lipopolysaccharide-stimulated human monocytes. *J Immunol.* 2007; 179(6):4065–4073. [PubMed: 17785845]
- Grunig G, Corry DB, Leach MW, Seymour BW, Kurup VP, Rennick DM. Interleukin-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergillosis. *J Exp Med.* 1997; 185(6):1089–1099. [PubMed: 9091582]
- Hartl D, Griesse M, Kappler M, Zissel G, Reinhardt D, Rebhan C, Schendel DJ, Krauss-Etschmann S. Pulmonary T(H)2 response in *Pseudomonas aeruginosa*-infected patients with cystic fibrosis. *J Allergy Clin Immunol.* 2006; 117(1):204–211. [PubMed: 16387607]
- Hartl D, Latzin P, Zissel G, Krane M, Krauss-Etschmann S, Griesse M. Chemokines indicate allergic bronchopulmonary aspergillosis in cystic fibrosis patients. *Am J Respir Crit Care Med.* 2006; 173(12):1370–6. 2006 Jun 15. [PubMed: 16543550]
- Haselmayer P, Grosse-Hovest L, von Landenberg P, Schild H, Radsak MP. TREM-1 ligand expression on platelets enhances neutrophil activation. *Blood.* 2007; 110(3):1029–1035. [PubMed: 17452516]
- Ho CC, Liao WY, Wang CY, Lu YH, Huang HY, Chen HY, Chan WK, Chen HW, Yang PC. TREM-1 expression in tumor-associated macrophages and clinical outcome in lung cancer. *Am J Respir Crit Care Med.* 2008; 177(7):763–770. [PubMed: 18096709]
- Hogaboam CM, Blease K, Mehrad B, Steinhäuser ML, Standiford TJ, Kunkel SL, Lukacs NW. Chronic airway hyperreactivity, goblet cell hyperplasia, and peribronchial fibrosis during allergic airway disease induced by *Aspergillus fumigatus*. *Am J Pathol.* 2000; 156:723–732. [PubMed: 10666400]
- Hogaboam CM, Blease K, Schuh JM. Cytokines and chemokines in allergic bronchopulmonary aspergillosis (ABPA) and experimental *Aspergillus*-induced allergic airway or asthmatic disease. *Front Biosci.* 2003; 8:e147–156. [PubMed: 12456354]
- Hogaboam CM, Gallinat CS, Taub DD, Strieter RM, Kunkel SL, Lukacs NW. Immunomodulatory role of C10 chemokine in a murine model of allergic bronchopulmonary aspergillosis. *J Immunol.* 1999; 162(10):6071–6079. [PubMed: 10229848]
- Holt PG, Macaubas C, Stumbles PA, Sly PD. The role of allergy in the development of asthma. *Nature.* 1999; 402(6760 Suppl):B12–17. [PubMed: 10586890]
- Imai T, Chantry D, Raport CJ, Wood CL, Nishimura M, Godiska R, Yoshie O, Gray PW. Macrophage-derived chemokine is a functional ligand for the CC chemokine receptor 4. *J Biol Chem.* 1998; 273(3):1764–1768. [PubMed: 9430724]

- Jakubzick C, Wen H, Matsukawa A, Keller M, Kunkel SL, Hogaboam CM. Role of CCR4 ligands, CCL17 and CCL22, during *Schistosoma mansoni* egg-induced pulmonary granuloma formation in mice. *Am J Pathol*. 2004; 165(4):1211–1221. [PubMed: 15466387]
- Joshi AD, Raymond T, Coelho AL, Kunkel SL, Hogaboam CM. A systemic granulomatous response to *Schistosoma mansoni* eggs alters responsiveness of bone-marrow-derived macrophages to Toll-like receptor agonists. *J Leukoc Biol*. 2008; 83(2):314–324. [PubMed: 18029396]
- Kauffman HF, Tomee JF, van de Riet MA, Timmerman AJ, Borger P. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. *J Allergy Clin Immunol*. 2000; 105(6 Pt 1):1185–1193. [PubMed: 10856154]
- Klesney-Tait J, Colonna M. Uncovering the TREM-1-TLR connection. *Am J Physiol Lung Cell Mol Physiol*. 2007; 293(6):L1374–L1376. [PubMed: 17934061]
- Knutsen AP, Mueller KR, Levine AD, Chouhan B, Hutcheson PS, Slavin RG. Asp f I CD4+ TH2-like T-cell lines in allergic bronchopulmonary aspergillosis. *J Allergy Clin Immunol*. 1994; 94(2 Pt 1):215–221. [PubMed: 7914901]
- Kurup VP, Guo J, Murali PS, Choi H, Fink JN. Immunopathologic responses to *Aspergillus* antigen in interleukin-4 knockout mice [see comments]. *J Lab Clin Med*. 1997; 130(6):567–575. [PubMed: 9422330]
- Kurup VP, Shen HD, Banerjee B. Respiratory fungal allergy. *Microbes Infect*. 2000; 2(9):1101–1110. [PubMed: 10967290]
- Kurup VP, Xia JQ, Rickaby DA, Dawson CA, Choi H, Fink JN. *Aspergillus fumigatus* antigen exposure results in pulmonary airway resistance in wild-type but not in IL-4 knockout mice. *Clin Immunol*. 1999; 90(3):404–410. [PubMed: 10075870]
- Montagnoli C, Fallarino F, Gaziano R, Bozza S, Bellocchio S, Zelante T, Kurup WP, Pitzurra L, Puccetti P, Romani L. Immunity and tolerance to *Aspergillus* involve functionally distinct regulatory T cells and tryptophan catabolism. *J Immunol*. 2006; 176(3):1712–1723. [PubMed: 16424201]
- Netea MG, Azam T, Ferwerda G, Girardin SE, Kim SH, Dinarello CA. Triggering receptor expressed on myeloid cells-1 (TREM-1) amplifies the signals induced by the NACHT-LRR (NLR) pattern recognition receptors. *J Leukoc Biol*. 2006; 80(6):1454–1461. [PubMed: 16940328]
- Nochi H, Aoki N, Oikawa K, Yanai M, Takiyama Y, Atsuta Y, Kobayashi H, Sato K, Tateno M, Matsuno T, Katagiri M, Xing Z, Kimura S. Modulation of hepatic granulomatous responses by transgene expression of DAP12 or TREM-1-Ig molecules. *Am J Pathol*. 2003; 162(4):1191–1201. [PubMed: 12651611]
- Paterson PJ, Seaton S, McHugh TD, McLaughlin J, Potter M, Prentice HG, Kibbler CC. Validation and clinical application of molecular methods for the identification of molds in tissue. *Clin Infect Dis*. 2006; 42(1):51–56. [PubMed: 16323091]
- Paterson PJ, Seaton S, McLaughlin J, Kibbler CC. Development of molecular methods for the identification of aspergillus and emerging moulds in paraffin wax embedded tissue sections. *Mol Pathol*. 2003; 56(6):368–370. [PubMed: 14645701]
- Radsak MP, Salih HR, Rammensee HG, Schild H. Triggering receptor expressed on myeloid cells-1 in neutrophil inflammatory responses: Differential regulation of activation and survival. *J Immunol*. 2004; 172(8):4956–4963. [PubMed: 15067076]
- Roilides E, Dimitriadou A, Kadiltsoglou I, Sein T, Karpouzas J, Pizzo PA, Walsh TJ. IL-10 exerts suppressive and enhancing effects on antifungal activity of mononuclear phagocytes against *Aspergillus fumigatus*. *J Immunol*. 1997; 158(1):322–329. [PubMed: 8977206]
- Schuh JM, Blease K, Kunkel SL, Hogaboam CM. Chemokines and cytokines: Axis and allies in asthma and allergy. *Cytokine Growth Factor Rev*. 2003; 14(6):503–510. [PubMed: 14563352]
- Schuh JM, Power CA, Proudfoot AE, Kunkel SL, Lukacs NW, Hogaboam CM. Airway hyperresponsiveness, but not airway remodeling, is attenuated during chronic pulmonary allergic responses to *Aspergillus* in CCR4^{-/-} mice. *FASEB J*. 2002; 16(10):1313–1315. [PubMed: 12154006]
- Skov M, Poulsen LK, Koch C. Increased antigen-specific Th-2 response in allergic bronchopulmonary aspergillosis (ABPA) in patients with cystic fibrosis. *Pediatr Pulmonol*. 1999; 27(2):74–79. [PubMed: 10088929]

Figure 1.

Soluble TREM-1 levels in serum and BAL, and whole lung (i.e., membrane-associated) TREM-1 levels at various times after conidia challenge alone in *Aspergillus* sensitized and *Conidia* challenged CBA/J mice. TREM-1 protein in all samples was measured using a specific ELISA. Data are mean $\pm$ SEM of $n = 4/5$ per group; *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 2.

Whole lung matrix metalloproteinase (MMP) mRNA levels at days 15 and 30 in the chronic fungal asthma model. Isolated RNA was analyzed using a quantitative PCR array for mouse MMPs. Data are mean $\pm$ SEM of $n = 4/5$ per group.

Figure 3.

Immunocytochemical detection of TREM-1 in bronchoalveolar lavage (BAL) cytopins at day 3 after conidia and Ad70- (A), AdDAP12- (B) or AdTREM-1 Ig (C & D) viral vector challenge. Goat anti-murine TREM-1 antibody (A, B & D) or control goat IgG antibody (C) was used to stain the BAL cytopsin samples. TREM-1 protein in whole lung tissue (E; upper panel) and BAL fluid (E; lower panel) was measured using a specific ELISA at day 7 after the intratracheal challenge. Quantitative analysis of DAP12 mRNA expression level was determined by TAQMAN analysis of whole lung tissue at day 3 after intratracheal challenge in all groups of mice (F). Images are representative of $n = 4/5$ per group, data are mean $\pm$ SEM of $n = 4/5$ per group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4.

Airway hyperresponsiveness (AHR) in Ad70-, AdDAP12- and AdTREM-1-treated groups at days 3, 7, and 14 days after viral vector and *A. fumigatus* conidia challenge in *A. fumigatus*-

sensitized mice (A). Peak increases in airway resistance or hyper-responsiveness (units = cm H₂O/ml/s) were determined at each time point after the intravenous injection of 210 µg/kg (A; upper panel) or 420 µg/kg (A; lower panel) methacholine. Adenovirus-induced DAP12 expression significantly diminished AHR at days 3, 7, and 14 after viral vector and conidia challenge in *A. fumigatus* sensitization. AHR values (units=cm H₂O/ml/s) in the saline-treated groups at day 7 were as follows: 5.8 ± 1.8 (210 µg/kg methacholine) and 18 ± 2.8 (420 µg/kg methacholine). At day 14, the AHR values (units = cm H₂O/ml/s) in the saline-treated group were as follows: 7 ± 1.1 (210 µg/kg methacholine) and 11 ± 3.8 (420 µg/kg methacholine).

Serum IgE (B; top left and bottom left panels) and IgG2A (B; top right and bottom right panels) levels at day 3 after viral vector and *A. fumigatus* conidia challenge in *A. fumigatus*-sensitized mice (both top panels) and at day 7 after viral vector *A. fumigatus* conidia challenge in *A. fumigatus*-sensitized mice and viral vector alone in non-sensitized mice (both bottom panels). Values are expressed as mean $\pm$ SEM; $n = 4-5$ /group/time point *P 0.05; **P 0.01; ***P 0.001

Figure 5.

TREM-1 blockade enhances airway remodeling. Representative photomicrographs of periodic acid Schiff (PAS)- (A–C) or Masson trichrome- (D–F) stained whole lung sections from Ad70 (A & D), AdDAP12 (B & E) and AdTREM-1 (C & F) treated mice at days 7 (A–C) or 28 (D–F) following intratracheal challenge with viral vectors and conidia. PAS stains mucins in goblet cells magenta and collagen is stained blue following Masson’s trichrome staining. Original magnification was 200×; representative photomicrographs were derived from a total of 10 mice per group from 2 independent experiments.

Figure 6.

TREM-1 is required for effective clearance of fungal material from the lungs of *A. fumigatus*-sensitized mice. Representative photomicrographs of whole lung sections stained with H&E (A–C) or GMS (D–F) from Ad70- (A & D), AdDAP12- (B & E), and AdTREM-1 Ig- (C & F) treated mice at day 7 after intratracheal viral vector and *Conidia* challenge. GMS stains fungal material black. Original magnification was 200 $\times$; representative photomicrographs were derived from a total of 10 mice per group from 2 independent experiments. (G) Quantitative PCR analysis of fungal DNA extracted from paraffin embedded whole lung tissue sections taken from *A. fumigatus*-sensitized mice at days 14, 21, and 28 after conidia challenge. Values are expressed as mean $\pm$ SEM; $n = 3$ –5/group/time point.

Figure 7.

ELISA analysis of cytokine and chemokine levels measured in whole lung samples from saline, Ad70-, AdDAP12-, and AdTREM-1 Ig-treated groups at days 7 (A), 14 (B), 21 (C & D), and 28 (E) after saline/viral vector and conidia challenge. Data are mean $\pm$ SEM; $n = 4-5/\text{group/timepoint}$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 8.

ELISA analysis of cytokine and chemokine levels measured in bronchoalveolar (BAL) samples from saline, Ad70-, AdDAP12-, and AdTREM-1 Ig-treated groups at days 3 (A), 7 (B), 14 (C), 21 (D), and 28 (E) after saline/viral vector and conidia challenge. Data are mean $\pm$ SEM; $n = 4-5$ /group/timepoint; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 9.

Quantitative PCR analysis of TREM-1 transcript levels in TLR2^{+/+} and TLR2^{-/-} bone marrow derived macrophages following TLR agonist activation (A). Quantitative PCR analysis of TREM-1 transcript levels in TLR2^{+/+} bone marrow macrophages exposed to soluble components from *A. fumigatus* in the presence of IgG control or a monoclonal anti-TLR2 antibody (B). Bone marrow was removed from *A. fumigatus*-sensitized mice at day 3 after the *Conidia* challenge and grown as described in the Materials and Methods section. Data are mean $\pm$ SEM; representative of 3 separate experiments; *P = 0.05.

Figure 10.

ELISA analysis of sTREM-1 (A) and IL-10 (B) levels in cell-free supernatants from bone marrow macrophages derived from *Aspergillus*-sensitized mice at day 3 after *Conidia* challenge (A) or naïve TLR2^{+/+} mice (B). TREM-1 and IL-10 protein in all samples were measured using specific ELISAs. Data are mean $\pm$ SEM; representative of 3 separate experiments; *P $\leq$ 0.05.