TREM-2 promotes macrophage survival and lung disease after respiratory viral infection

Kangyun Wu,1 Derek E. Byers,1 Xiaohua Jin,1 Eugene Agapov,1 Jennifer Alexander-Brett,1 Anand C. Patel,1,2 Marina Cella,3 Susan Gilfilan,3 Marco Colonna,3 Daniel L. Kober,1 Tom J. Brett,1,4,5 and Michael J. Holtzman1,5

1Pulmonary and Critical Care Medicine, Department of Medicine, 2Department of Pediatrics, 3Department of Pathology and Immunology, 4Department of Biochemistry and Biophysics, and 5Department of Cell Biology, Washington University School of Medicine, St. Louis, MO 63110

Viral infections and type 2 immune responses are thought to be critical for the development of chronic respiratory disease, but the link between these events needs to be better defined. Here, we study a mouse model in which infection with a mouse parainfluenza virus known as Sendai virus (SeV) leads to long-term activation of innate immune cells that drive IL-13–dependent lung disease. We find that chronic postviral disease (signified by formation of excess airway mucus and accumulation of M2-differentiating lung macrophages) requires macrophage expression of triggering receptor expressed on myeloid cells-2 (TREM-2). Analysis of mechanism shows that viral replication increases lung macrophage levels of intracellular and cell surface TREM-2, and this action prevents macrophage apoptosis that would otherwise occur during the acute illness (5–12 d after inoculation). However, the largest increases in TREM-2 levels are found as the soluble form (sTREM-2) long after clearance of infection (49 d after inoculation). At this time, IL-13 and the adapter protein DAP12 promote TREM-2 cleavage to sTREM-2 that is unexpectedly active in preventing macrophage apoptosis. The results thereby define an unprecedented mechanism for a feed-forward expansion of lung macrophages (with IL-13 production and consequent M2 differentiation) that further explains how acute infection leads to chronic inflammatory disease.

A critical step toward improved diagnosis and treatment of chronic inflammatory diseases depends on defining the immune mechanisms for the persistent accumulation of activated immune cells in the target tissue. In the case of the lung, clinical evidence suggests that acute infection with a respiratory virus might lead to chronic lung diseases such as asthma and COPD (Holtzman, 2012). To determine precisely how acute infection causes chronic lung disease, we developed a high-fidelity mouse model of this process. In this model, mouse parainfluenza virus (also known as Sendai virus, SeV) is substituted for the related human pathogen to achieve more efficient viral replication and thereby produce the severe acute illness and subsequent chronic respiratory disease that is typical of the pathology found in humans (Walter et al., 2002). Using this model system, we determined that postviral lung disease depends on airway progenitor epithelial cell (APEC) production of IL-33 to drive invariant NK T cells (iNKT cells) and lung macrophages toward IL-13 production (Kim et al., 2008; Byers et al., 2013). The result is IL-13–dependent inflammation (signified by type 2 activation and accumulation of lung macrophages) and airway mucus production (signified by MUC5AC mucin gene expression). This innate epithelial to immune cell loop also appears relevant to human disease because increased numbers of IL-33–expressing APECs are found in association with an IL-13 gene expression signature (including increased MUC5AC mRNA and protein) in the lungs of humans with severe chronic obstructive pulmonary disease (COPD; Kim et al., 2008; Agapov et al., 2009; Alevy et al., 2012; Byers et al., 2013).

In our previous work, we recognized that the APEC population was capable of self-renewal
and inducible release of IL-33 to sustain ongoing activation of the innate immune system (Holtzman et al., 2014). However, the existing data did not explain the selective activation of the lung macrophage population and the special dominance of type 2 (M2) macrophages as a downstream part of the disease process. In the present study, we therefore aimed to better understand how the lung macrophage component of this disease process is triggered by acute infection and then is manifest for months. We reasoned that triggering receptor expressed on myeloid cells 2 (TREM-2) might contribute to this process because M2 polarization is associated with TREM-2 expression in isolated macrophages (Turnbull et al., 2006). In pursuing this possibility, we found that the soluble form of TREM-2 (sTREM-2) was linked to the development of chronic postviral lung disease and was active in promoting macrophage survival. The data stand in contrast to the conventional view that cleavage of cell surface TREM-2 to sTREM-2 results in an inactive end product. The results thereby provide for a previously unrecognized control over macrophage survival and a consequent type 2 immune response that can serve both as a pathogenic mechanism and as a therapeutic target and accompanying biomarker for chronic inflammatory disease.

RESULTS
Macrophage control of postviral disease
To further define the role of macrophages in our postviral mouse model of chronic lung disease (Walter et al., 2002), we first assessed the impact of a new strategy for macrophage deficiency. We previously showed that mice that were treated with clodronate or mice that were homozygous for the op mutation in the Csf1 gene (Csf1op/op) led to marked macrophage depletion and blockade of postviral lung disease, but both pharmacologic and genetic approaches to depletion led to mice that were also more susceptible to viral infection (Tyner et al., 2005; Kim et al., 2008). In the present case, we started with Csf1op/op T (op/op T) mice, which carry an osteocalcin-driven Csf1 transgene (T) that rescues the osteopetrotic, but not the macrophage defect, in op/op mice (Abboud et al., 2002). We then used these mice to generate heterozygous Csf1wt/op T (wt/op T) mice with a subtler defect in macrophage level and perhaps function. Of note, we still found that the increase in lung levels of tissue monocytes (SSChighLy6G−F4/80−CD11b+) and interstitial macrophages (SSCmediumCD11cLy6G−Siglec-F−F4/80−CD11b+) that occurs in WT (wt/wt) mice at 5 d post-inoculation (dpi) with Sendai virus (SeV) was significantly attenuated in wt/op T mice (Fig. 1 B). Tissue monocytes, which were the most abundant monocyte–macrophage population in the lung, exhibited no increase in wt/op T mice at 49 dpi. In concert with the decreased lung monocyte–macrophage levels in wt/op T mice, we also found that induction of IL-13 expression and mucosal cell metaplasia in wt/wt mice was markedly inhibited in wt/op T mice at 49 dpi (Fig. 1 C). Similarly, the postviral increases in lung levels of Il13 mRNA and downstream Muc5ac mRNA and type 2 macrophage (M2) markers Arg1 and Chitinase 3–like-3 (Chi3l3) mRNA in wt/wt mice were completely blocked in wt/op T mice at 49 dpi (Fig. 1 D). These findings are consistent with our previous work showing that lung macrophages are critical for IL-13 production, and in turn, that IL-13 is required to drive mucus production and M2 differentiation in our postviral mouse model of lung disease (Tyner et al., 2006; Kim et al., 2008; Byers et al., 2013). The results also provide a basis for next determining what factors might regulate the expansion of disease-producing lung macrophages under these conditions.

TREM-2 control of postviral disease
Based on the association of TREM-2 with M2 differentiation (Turnbull et al., 2006), we investigated the role of TREM-2 in macrophage-dependent postviral lung disease. In that context, we found that the development of this type of lung disease was associated with increased lung Trem2 (but not other Trem) mRNA levels based on gene expression microarray and real-time qPCR assay (Fig. 2, A and B; and Table S1). Moreover, the development of postviral lung disease at 49 dpi in WT mice was markedly attenuated in Trem2−/− mice. Thus, Trem2−/− mice showed marked decreases in lung inflammation and airway mucus production based on tissue staining (Fig. 2 C) as well as significantly decreased lung levels of the key cytokine Il13 mRNA, the predominant mucin Muc5ac mRNA, and the M2 markers Arg1 and Chi3l3 mRNA based on real-time qPCR assay (Fig. 2 D). Dap12−/− mice exhibited a similar inhibition of postviral disease (Fig. 2, C and D), consistent with the association of TREM-2 and DAP12 phenotype in other systems (Bouchon et al., 2000; Kaifu et al., 2003; Turnbull et al., 2006). In contrast, Trem1/3−/− mice manifested no significant difference in the development of chronic postviral disease (Fig. 2, C and D), perhaps divergent from the pro-inflammatory role of TREM-1 in other disease models (Bouchon et al., 2001a; Schenk et al., 2007; Klesney-Tait et al., 2013).

The distinct nature of TREM-2 actions in postviral lung disease prompted a further examination of mechanism. We
Figure 1. Effect of macrophage deficiency on postviral lung disease. (A) Lung levels of total cells, tissue monocytes (tissue monos), interstitial macrophages (interstitial macs), and alveolar macrophages (alveolar macs) for wt/wt and wt/opT mice at 5 dpi with SeV or UV-inactivated SeV (SeV-UV) determined by flow cytometry staining with fluorescent bead-based quantitation. (B) For conditions in (A), corresponding values for 49 dpi. (C) Representative photomicrographs of lung sections stained with PAS plus hematoxylin, immunostained for MUC5AC and counterstained with hematoxylin, or immunostained for IL-13 and counterstained with DAPI. Bars, 200 µm. (D) Lung levels of Il13, Muc5ac, Arg1, and Chi3l3 mRNA in wt/wt and wt/opT mice at 49 dpi with SeV or SeV-UV. For A, B, and D, * represents a significant increase from SeV-UV, and ** represents a significant decrease from corresponding wt/wt control value. All experimental data were verified in at least three independent experiments.
Figure 2. Induction and function of Trem2 gene expression during postviral lung disease. (A) Gene expression microarray analysis of mRNA from lungs of mice at 3 and 49 dpi with SeV (10^5 pfu) or SeV-UV. Scatter plot depicts log2 normalized gene expression. Each symbol represents the expression value for an individual gene with the top 20 differentially expressed genes colored red and annotated in black, and others colored gray. Genes encoding Trem family members and Dap12 are colored red if significantly increased, blue if decreased, or white if unchanged, and are annotated in red. (B) Lung levels of Trem1, Trem2, and Il13 mRNA at indicated dpi with SeV. * represents a significant increase from (-) SeV control value. Values at 0 dpi were no different than those for SeV-UV 0–49 dpi. (C) Representative photomicrographs of PAS and Muc5c staining of lung sections from WT, Trem2^-/-,
first established that there was no significant difference in acute illness among $\text{Trem2}^{-/-}$, $\text{Dap12}^{-/-}$, and $\text{Trem1/3}^{-/-}$ mice as signified by similarities in body weight change, viral titer and tissue distribution, and pattern of tissue inflammation (Fig. 3, A–D). Therefore, we returned to our interest in the role of TREM-2 in modifying long-term macrophage function. We first found that TREM-2 expression at 49 dpi was specific to lung tissue macrophages based on co-localization with macrophage markers $\text{F}4/80$ and $\text{Mac-3}$ in tissue immunostaining (Fig. 4 A). Similarly, TREM-2 expression was found in lung macrophages (CD45$^+$F4/80$^+$) and not stromal cells, e.g., epithelial cells (CD45 EpCAM$^+$), based on flow cytometry staining (unpublished data).

As demonstrated previously (Kim et al., 2008), we also found a marked expansion of IL-13$^+$ lung macrophages in WT mice at 49 dpi (Fig. 4 B). Here, we show that this expansion is markedly down-regulated in $\text{Trem2}^{-/-}$ mice based on tissue immunostaining (Fig. 4 B). In addition, FACS-based separation of lung macrophage populations revealed that tissue monocytes and interstitial macrophages exhibited a marked increase in $\text{Il13}$, $\text{Arg1}$, and $\text{Chi3l3}$ mRNA levels with the development of postviral disease at 49 dpi in WT mice, and this postviral induction was markedly attenuated in $\text{Trem2}^{-/-}$ mice (Fig. 4 C). In contrast, alveolar macrophages showed no induction of $\text{Il13}$ mRNA but nonetheless still manifested a slight increase in $\text{Arg1}$ mRNA and a marked increase in $\text{Chi3l3}$ mRNA at 49 dpi in WT mice. The observed levels of $\text{Il13}$, $\text{Arg1}$, and $\text{Chi3l3}$ mRNA in alveolar macrophages from WT mice were nearly identical to those in $\text{Trem2}^{-/-}$ mice (Fig. 4 C). This subset profile might again reflect a predominant
Moreover, flow cytometry staining for TREM-2 was found exclusively on CD11b+CD11c– lung cells at 5 dpi (Fig. S2), which is consistent with localization to tissue monocytes and interstitial macrophages and not to alveolar macrophages or dendritic cells. Postviral induction of cell surface TREM-2 was found only at 2–8 dpi (Fig. 5, A and B) and was therefore similar to induction for lung levels of SeV, suggesting that SeV replication might increase cell surface TREM-2 on macrophages. To follow up this possibility, we studied BMDMs infected with GFP-labeled SeV and showed an increase in cell surface staining for TREM-2 that correlated closely with viral titer (Fig. 5, C and D). Moreover, SeV infection caused a dose-dependent increase in cell surface TREM-2 levels on BMDMs that was reproduced using poly(IC) treatment, effect of TREM-2 deficiency on tissue monocytes and interstitial macrophages. Given the predominance of the tissue monocyte population, these findings were consistent with the decrease in whole lung levels of Il13, Arg1, and Chi3l3 mRNA found in Trem2-/- mice.

Cell surface TREM-2 expression and function
We next assessed the regulation of TREM-2 expression and function under the current paradigm that TREM-2 expressed on the cell surface works in coordination with its adapter protein DAP12 to signal for macrophage activation (Turnbull et al., 2006). In the present model, cell surface expression of TREM-2 on lung macrophages was detected on tissue monocytes and interstitial macrophages but not alveolar macrophages (Fig. 5 A). Moreover, flow cytometry staining for TREM-2 was found exclusively on CD11b+CD11c– lung cells at 5 dpi (Fig. S2), which is consistent with localization to tissue monocytes and interstitial macrophages and not to alveolar macrophages or dendritic cells. Postviral induction of cell surface TREM-2 was found only at 2–8 dpi (Fig. 5, A and B) and was therefore similar to induction for lung levels of SeV, suggesting that SeV replication might increase cell surface TREM-2 on macrophages. To follow up this possibility, we studied BMDMs infected with GFP-labeled SeV and showed an increase in cell surface staining for TREM-2 that correlated closely with viral titer (Fig. 5, C and D). Moreover, SeV infection caused a dose-dependent increase in cell surface TREM-2 levels on BMDMs that was reproduced using poly(IC) treatment,
Figure 5. Regulation of cell surface and intracellular TREM-2 levels during viral replication and postviral lung disease. (A) Representative histograms from FACS analysis of TREM-2 immunostaining for tissue monos, interstitial macs, and alveolar macs from lungs of mice at 5 or 49 dpi with SeV-UV or SeV. (B) Corresponding MFI values for cell surface TREM-2 levels on tissue monos at the indicated dpi based on analysis conditions in A. (C) Representative cytograms from flow cytometry analysis of cell surface TREM-2 immunostaining for BMDMs at 0–3 dpi with GFP-expressing SeV (SeV-GFP). (D) Corresponding MFI values for cell surface TREM-2 and SeV-GFP for conditions in C. (E) Representative histograms for flow cytometry analysis of cell surface and intracellular TREM-2 immunostaining for BMDMs with indicated conditions. (F) Western blotting of TREM-2 in BMDMs at 2 dpi with SeV (MOI 0–10). (G) ELISA of cellular TREM-2 and sTREM-2 in BMDMs at 2 dpi with SeV for indicated MOI or with SeV at MOI 10 for 0–3 dpi. * represents a significant increase from corresponding control at MOI 0 and 0 dpi and Ø represents an undetectable level of sTREM-2. (H) Trem2 mRNA level in BMDMs with SeV at MOI 1 and 10 for 0–3 dpi. All experimental data verified in at least three independent experiments.
and this increase was unaffected by DAP12, TLR3, or PKR deficiency (Fig. 5 E). In addition to the increase in surface TREM-2 levels, we also found that SeV infection caused a smaller but sustained increase in intracellular levels of TREM-2 in BM-derived macrophages (BMDMs) unaccompanied by any detectable production of sTREM-2 or any increases in Trem2 mRNA (Fig. 5, F–H). A similar increase in intracellular TREM-2 level was found in vivo at 5 and 49 dpi. Together, the results suggest that SeV promotes an increase in surface TREM-2 levels based on increased TREM-2 formation and either transport to or retention at the cell surface with relatively little if any TREM-2 cleavage from the cell surface.

The relatively transient increase in cell surface TREM-2 levels suggested an acute effect on macrophage function after viral infection. Consistent with this idea, we found that the increase in the number of tissue monocytes (and interstitial macrophages but not neutrophils) at 5 dpi in WT mice was attenuated in Trem2−/− mice (Fig. 6 A and not depicted). This attenuation was not accompanied by decreases in monocyte chemokine production based on lung levels of Ccl2 and Cx3cl1 mRNA at 3–5 dpi (Fig. 6 B) or decreases in monocyte influx into the lung based on tracking CD45.2 transferred monocytes by flow cytometry at 5 dpi (Fig. 6 C). However, we did detect increased macrophage apoptosis in Trem2−/− mice compared with WT mice based on immunostaining for active caspase-3 and Mac-3 at 5–12 dpi (Fig. 6, D and E), suggesting a role for TREM-2 in specifically promoting macrophage survival after viral infection. Cells that were active caspase-3+ were not also SeV+ (Fig. 6 F), suggesting that TREM-2 antagonized apoptosis actions independent of viral cytopathic effect. In addition, the timing of apoptosis corresponded to a relative decrease in Csf1 mRNA and CSF-1 protein levels in the lung (Fig. 6, G and H), consistent with the link between CSF-1 and macrophage survival via DAP12 signaling (Otero et al., 2009). Moreover, we detected no defect in CSF-1 production in Trem2−/− mice suggesting that the CSF-1 survival signal required downstream activation of TREM-2 to prevent apoptosis. Indeed, we found a marked increase in apoptosis with CSF-1 withdrawal in BMDMs from Trem2−/− mice compared with WT control mice based on propidium iodide staining, Annexin V staining, and active Caspase-3 immunoblotting (Fig. 6, I–K). We detected a slight increase in apoptosis in BMDMs from Trem2−/− mice even in cultures with CSF-1, but this effect was also attributable to CSF-1 withdrawal because CSF-1 was added only once at the start of the 2-d culture period and was therefore being consumed and depleted from the culture medium. This condition therefore represents a partial degree of CSF-1 withdrawal and a consequent loss of the CSF-1 to TREM-2 survival signal in Trem2−/− cells. Together, our results suggested that cell surface TREM-2 might preserve macrophage survival and promote macrophage accumulation after viral infection and thereby lead to long-term macrophage-dependent disease. However, the relatively transient effect of TREM-2 during acute infection also raised the possibility that TREM-2 had additional effects at later time points that influenced long-term disease outcome.

sTREM-2 expression and function

To pursue the possible chronic actions of TREM-2 in the postviral model, we first determined whether there was long-term production of TREM-2 protein in the lung in this model. Indeed, we found a marked increase in lung levels of Trem2 mRNA at 49 dpi compared with baseline or to the relatively small increase found at 5 dpi (Fig. 7 A). Given the absence of detectable cell surface TREM-2 at 49 dpi and the relatively small increase in intracellular TREM-2 at either 5 or 49 dpi (Fig. 5 A), this late increase in Trem2 mRNA levels at 49 dpi was likely a marker of sTREM-2 formation in the diseased lung. Since IL-13 production was also markedly increased at 49 dpi, we questioned whether IL-13 might promote TREM-2 cleavage at the cell surface and consequent formation of sTREM-2. In support of this possibility, we found that IL-13 administration caused a time-dependent loss of cell surface levels of TREM-2 in BMDMs (Fig. 7 B). The effect of IL-13 was specific for the IL-13 receptor since the same effect was found for IL-4 (that also activates this receptor) but was not detectable for other type 2 cytokines (IL-5 and IL-9; Fig. 7 C). Treatment with IL-13 also resulted in a time-dependent increase in cellular TREM-2 levels in BMDMs, as expected for M2 differentiation, but caused an even greater increase in sTREM-2 levels released from BMDMs (Fig. 7 D). The increases in cellular TREM-2, especially the even greater increases in sTREM-2 production, were attenuated in BMDMs from Dap12−/− mice (Fig. 7 E). Moreover, the expected increase in sTREM-2 levels was not found during postviral disease in Dap12−/− mice (Fig. 7 E). Together, these findings indicated that both IL-13 and DAP12 were contributing to sTREM-2 formation and any consequent function of sTREM-2 in chronic postviral disease.

To pursue a possible role for sTREM-2 in postviral disease, we analyzed the effect of recombinant sTREM-2 (amino acids 19–136) on macrophage function. Immunostaining of BMDMs incubated with sTREM-2 showed that the protein was localized to the cell cytoplasm and not the cell surface and was thereby accessible to intracellular proteins (Fig. 8 A). In that context, we found that sTREM-2 administration to BMDMs caused a significant increase in Arg1 mRNA levels (as a sign of M2 differentiation), but the increase was not preserved for other M2 markers (e.g., Chil3 mRNA) and was not nearly as effective as IL-13 for driving these responses (Fig. 8 B). Thus, the direct effect of sTREM-2 on M2 differentiation was relatively small and was therefore unlikely to contribute to biological function or postviral disease. However, in the process of conducting these experiments, we recognized that sTREM-2 treatment of BMDMs resulted in marked protection against the usual progression to cell death in this culture system, and this protective effect resulted in expanded numbers of BMDMs from both WT and Trem2−/− mice (Fig. 8 C). Similarly, sTREM-2 treatment caused a dose-dependent expansion of BMDMs from WT, Trem2−/−, and Dap12−/− mice (Fig. 8 D). Here again,
Figure 6. Effect of cellular TREM-2 on macrophage apoptosis during acute viral infection. (A) Flow cytometry analysis of lung levels of total cells, tissue monos, and neutrophils (SSC⁻Ly6G⁻) for WT and Trem2⁻/⁻ mice at 0–5 dpi with SeV. (B) Ccl2 and Cx3cl1 mRNA levels in WT and Trem2⁻/⁻ mice at 0–5 dpi. For A and B, values represent mean ± SEM for 5 mice, and * represents a significant increase from 0 dpi and ** a significant decrease from corresponding WT mice. (C) Representative cytograms for flow cytometry analysis of levels of CD45.2⁺ tissue monos in WT and Trem2⁻/⁻ mice at 3 dpi after CD45.2 WT and Trem2⁻/⁻ BMDMs were introduced into CD45.1 WT mice at 0 dpi. (D) Representative photomicrographs of active caspase-3 and Mac-3 immunostaining of lung sections from WT and Trem2⁻/⁻ mice at 8 dpi. Arrows indicate cells with double-positive immunostaining. Bar, 200 µm. (E) Quantification of analysis in (D). Values represent mean ± SEM for 12 randomly selected fields of lung sections from 3 mice, and ** represents a significant increase from corresponding WT mice. (F) Representative photomicrographs of active caspase-3 and SeV immunostaining of lung sections from WT and Trem2⁻/⁻ mice at indicated dpi. (G) Lung levels of Csf1 mRNA for WT and Trem2⁻/⁻ mice at indicated dpi. For G and H, values represent mean ± SEM for 5 mice, and * represents a significant increase from 0 dpi. (H) Flow cytometry analysis of propidium iodide staining of BMDMs from WT and Trem2⁻/⁻ mice cultured with or without CSF-1 for 1 d. (I) Levels of Annexin V immunostaining in PI⁻ cells for culture conditions in (H). For G and H, values represent mean ± SEM for 6 randomly selected fields of replicate wells, and * represents a significant increase from (+) CSF-1, and ** a significant increase from corresponding WT mice. (K) Western blot levels of active caspase-3 for culture conditions in I. All experimental data were verified in at least three independent experiments.
the baseline numbers of BMDMs were increased in WT mice compared with Trem2−/− and Dap12−/− mice under these culture conditions, but this difference was corrected in response to sTREM-2 treatment (Fig. 8 D). The protective effect was specific for active sTREM-2 because it was lost with heat inactivation (Fig. 8 E). Flow cytometry analysis with propidium iodide staining indicated that sTREM-2 administration resulted in attenuation of apoptosis due to CSF-1 withdrawal in BMDMs from WT mice, as well as Trem2−/− and Dap12−/− mice (Fig. 8 F). As expected, the level of apoptosis with CSF-1 withdrawal in BMDMs from WT mice was markedly increased in Trem2−/− and Dap12−/− mice, but this effect was also fully preventable and dose-dependent with sTREM-2 administration (Fig. 8 F). Treatment with sTREM-2 also reversed the increase in active caspase-3 levels that develops with CSF-1 withdrawal, confirming an effect on apoptosis (Fig. 8 G). In contrast, sTREM-2 had no effect on cellular proliferation based on BrdU incorporation (Fig. 8 H), as expected for cell cultures deprived of CSF-1 stimulation. This result is also consistent with the absence of significant cell proliferation in the lung in vivo when disease becomes manifest (Tyner et al., 2006). Together, these findings support a role for sTREM-2 in protection against macrophage apoptosis under low CSF-1 conditions, and thereby explained the increased levels of apoptosis under these conditions in Trem2−/− and Dap12−/− macrophages that are unable to generate sTREM-2.

Given the difficulties in monitoring apoptosis in vivo (e.g., we detected no active caspase-3 tissue staining at 49 dpi; unpublished data) we sought additional markers of sTREM-2 action during postviral lung disease. In that regard, we were able to establish that sTREM-2 treatment resulted in a time-dependent and selective increase in molecular pathways connected to cell survival and proliferation, e.g., ERK1/2 and MAPK14 phosphorylation but not AKT phosphorylation or BCL-2 induction (Fig. 9 A). The effect of sTREM-2 on phospho-ERK1/2 and phospho-MAPK formation was more pronounced in BMDMs from Trem2−/− mice compared with WT mice (Fig. 9 A), consistent with the enhanced effect of sTREM-2 on viability of BMDMs from Trem2−/− compared with WT mice based on fold-change (Fig. 8 D). In translating these findings to the postviral model in vivo, we found that phospho-ERK1/2 immunostaining was markedly increased and co-localized to lung macrophages at 49 dpi compared with 5 dpi or SeV-UV control conditions in WT mice (Fig. 9 B). Moreover, the increase in phospho-ERK1/2 immunostaining was not detected at 49 dpi in Trem2−/− mice (Fig. 9 B). These results were consistent with increases in the numbers of total
immune cells and macrophages in the lung at 49 dpi in WT mice, as well as marked inhibition of this cellular response at 49 dpi in Trem2−/− mice (Fig. 9 C). Together, these findings provide for a crucial regulatory role of TREM-2 in a feed-forward pathway that expands the M2 macrophage population and thereby promotes IL-13–dependent chronic lung disease.
in the lung. In the context of our previous work, the increased number of macrophages would be available to interact with iNKT cells and drive further IL-13 production thereby resulting in IL-13–dependent differentiation of the macrophage population toward an M2 pattern of gene expression (including Arg1 and Chi3l3) and differentiation of an airway progenitor epithelial cell (APEC) niche to airway mucous cells (AMCs) as signatures of chronic lung disease (as diagrammed in Fig. 10). The scheme is consistent with our observation that TREM-2–deficient mice are markedly protected from the development of chronic lung disease as a long-term consequence of viral infection.

The role of TREM-2 in chronic disease described in this study provides for at least four major and unexpected insights. First, TREM-2 signaling was often portrayed as antiinflammatory and reparative, whereas increased TREM-1 signals

Figure 9. Effect of TREM-2 deficiency on apoptosis signaling pathway. (A) Western blot levels of indicated cell survival proteins in BMDMs cultured from WT and Trem22/2 mice after treatment with mouse sTREM-2 (200 ng/ml) for 0–24 h. (B) Representative photomicrographs of phospho-ERK1/2 (p-ERK) and Mac-3 immunostaining with DAPI counterstaining of lung sections from WT and Trem22/2 mice at 5 or 49 dpi with SeV or SeV-UV. Arrows indicate cells with double-positive immunostaining. Bar, 200 µm. (C) Flow cytometry analysis of lung levels of total cells and tissue monos for WT and Trem22/2 mice at 0 and 49 dpi. Values represent mean ± SEM for 5 mice, and * represents a significant increase from 0 dpi and ** a significant decrease from corresponding WT mice. Values at 0 dpi were no different than those for SeV-UV (49 dpi). All experimental data were verified in at least three independent experiments.
were thought to be proinflammatory and destructive in models of intestinal and CNS disease (Schenk et al., 2007; Takahashi et al., 2007; Seno et al., 2009). This view is consistent with studies showing that engagement of TREM-1 promotes secretion of proinflammatory cytokines (including IL-6 and TNF) and increases the inflammatory responses downstream of TLR and NLR engagement (Bouchon et al., 2000, 2001a; Bleharski et al., 2003; Netea et al., 2006; Fortin et al., 2007; Schenk et al., 2007). Meanwhile, similar approaches showed that TREM-2 activation generally antagonized activation signals for inflammation (Daws et al., 2001; Colonna, 2003; Hamerman et al., 2006; Turnbull et al., 2006). In contrast, the present results provide for a paradigm in which TREM-2 activation promotes the development of chronic inflammatory disease. This model is consistent with reports for up-regulation of M2 gene expression (including TREM2) in macrophages isolated from cigarette smokers with and without COPD (Shaykhiev et al., 2009; Koth et al., 2010). However, these previous studies as well as others that assess association with disease cannot determine cause and effect actions of TREM signaling in the development of disease. Moreover, the present application of new tools to separately study both TREM-1 and TREM-2 deficiency in vivo and in vitro in the same model system was critical to dissect any differences in TREM family member function.

A second unexpected aspect of the present findings is the biological activity of sTREM-2. In fact, we initially approached the issue of TREM-2 activity as being a function of TREM-2 localization to the cell surface, where it could presumably bind to an as yet poorly defined ligand, and in association with DAP12, initiate a signaling pathway for cell survival (Otero et al., 2009). Indeed, we were able to secure evidence for this mechanism in the setting of acute infection, wherein viral replication increases TREM-2 on the surface of infected macrophages. However, this relatively transient effect on macrophage survival did not appear to explain the long-term effect of TREM-2 on postviral lung disease. We therefore pursued evidence that the greatest increases in TREM-2 lung levels were found long after clearance of infectious virus and were manifest as marked increases in sTREM-2 that had been cleaved from the cell surface. These circumstances led to the unexpected function of sTREM-2 as a potent antiapoptotic factor for macrophages. Our data suggests that sTREM-2 acts as an intracellular messenger to trigger the activation of kinases (e.g., ERK1/2) that promote cell survival. However, additional work will need to define the precise molecular pathway downstream of sTREM-2 actions, including those proposed for TREM-2–DAP12 signaling for cell survival (Otero et al., 2009, 2012). Meanwhile, however, our results may also help to explain the proposed benefit of sTREM-2 cleavage in neurodegenerative disease, where decreased sTREM-2 cleavage caused by mutation may be associated with impaired macrophage (i.e., microglial) function in the CNS (Kleinberger et al., 2014).

A third unanticipated result of our study was the potent role of DAP12 and IL-13 in regulating TREM-2 cleavage and sTREM-2–DAP12 formation. Similar to signaling function of cell surface TREM-2, the formation of sTREM-2 and any consequent downstream action of sTREM-2 was still dependent on DAP12. This new connection between sTREM-2 and DAP12 could help explain how distinct TREM signals are transduced into different downstream effects on cell behavior. Thus, both TREM-1 and TREM-2 on the cell surface are proposed to signal via DAP12, and this adaptor has only a single immunoreceptor tyrosine-based activation motif (ITAM) for subsequent signal transduction. It has been proposed that the avidity of TREM (or other receptor) interaction with DAP12 might dictate a positive versus negative signal from this ITAM to the downstream kinase (Turnbull and Colonna, 2007). In the present experiments, we find that TREM-2 function is linked to DAP12 by a protein maturation process that appears distinct from TREM-1–DAP12 and TREM-2–DAP12 signaling. Similarly, IL-13 also contributed to the TREM-2 cleavage process in what appears to be a novel role for IL-13. In both cases, additional work is needed to define the molecular mechanism for TREM-2 cleavage. This type of
sTREM-2 formation or action. Initial studies already suggest an increase in TREM-2 expression in concert with excess levels of airway mucus and M2 macrophages in human lung disease (Byers and Holtzman, 2011; Byers et al. 2013. International Meeting of the American Thoracic Society. Abstr. A141.). The present findings justify further validation of these observations in humans with chronic lung disease and additional work to correct any abnormalities in TREM-2 action in this type of disease and other diseases that implicate a role for TREM-2 in regulating macrophage survival.

MATERIALS AND METHODS

Mice. WT C57BL/6j mice were obtained from The Jackson Laboratory. The op/opT mice were generated as described previously (Abboud et al., 2002) and were provided by N. Ghosh-Choudhury (University of Texas, San Antonio). These mice were crossed to WT mice to generate heterozygous wt/opT mice and littermate wt/wt control mice. Dap12+/− mice were provided by T. Takai (Tohoku University, Sendai, Japan). Trem2+/− mice were generated as described previously (Turnbull et al., 2006). Trem1/−/− mice were generated as described previously and were backcrossed to C57BL/6 mice until >99% of the loci were derived from this strain. Th3−/− mice were generated as described previously (Alexopoulou et al., 2001). Pkr−/− mice were generated as described previously (Yang et al., 1995) and rederived from frozen embryos obtained from M. White and H. Virgin IV (Washington University, St. Louis, MO). All mice were maintained on a C57BL/6j genetic background and were used in protocols approved by Animal Studies Committee of Washington University School of Medicine in accordance with National Institutes of Health guidelines.

Viral inoculation and monitoring. Mice were inoculated with SeV (10⁴ pfu), an equivalent amount of UV-inactivated SeV (SeV-UV), or PBS as described previously (Kim et al., 2008). SeV-UV and PBS inoculations gave identical results, and therefore are treated interchangeably as controls. Viral titers for stock solutions and lung infections were monitored by plaque-forming and real-time PCR assays as described previously (Kim et al., 2008).

Analysis of mRNA. For gene expression microarrays, RNA was isolated and analyzed using Illumina Mouse-WG6 v2 beadChips (Illumina) as described previously (Byers et al., 2013). Visualization and plotting was performed using DecisionSite for Functional Genomics (TIBCO Spotfire). Microarray data were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) under series accession nos. GSE49603 and GSE61437. For real-time qPCR assays, RNA was purified from lung homogenate using TRIzol (Invitrogen) and was converted to cDNA using the High-Capacity cDNA Archive kit (Applied Biosystems). Target mRNA was quantified by real-time PCR assay using specific fluorogenic probes and primer sets and the Fast Universal PCR Master Mix system (Applied Biosystems). Primer-probe sets for mouse Ccl2 (Mm00441242_m1), Cx3cr1l (Mm00436454_m1), Trem1 (Mm00451736_m1), Trem2 (Mm00451744_m1), Chi3l3 (Mm00657889_mH), Arg1 (Mm00475988_m1), and Dap12 (Mm0049152_m1) were obtained from Applied Biosystems. The forward and reverse primers and probes for mouse Il13 were 5′-GGTCCCAAGATCGTGTGTCCT-3′, 5′-CCACACTCCATACCATGCGTGCTG-3′, and 5′-AAAGCCAGACTCCCTGGTGCCAC-3′; for Muc5ac were 5′-TACCCTCTCCCTGCTTCTGACGGCAGGTGCTA-3′, 5′-ATAGTAAACGTGCGCAATACAGTGTCCTGCT-3′, and 5′-TATACCCCTTGAGACTCCATCCTACA-3′; for Csf1 were 5′-CCTTCAGTTGCGACCTGCCTT-3′, 5′-TGATTGGGCTTCCACTGTTTCT-3′, and 5′-CATTAATGGCTTCGCTCTGCAC-3′; for Gapdh were 5′-AAGGTGAAGGTCGGGTAACAA-3′, 5′-CAGCTGATGACGCTCTTCCACC-3′, and 5′-CGATGGTGCATTGCTTGGGCGC-3′.
probes were designed to span introns and did not react with genomic DNA. Samples were assayed on the 7500 Fast Real-Time PCR System and analyzed using the 7500 Fast System Software (Applied Biosystems). Levels of specific gene expression were normalized to Gapdh mRNA levels.

**Western blotting.** For TREM-2 expression, BMDMs were seeded in 6-well tissue culture plates and were infected with SeV at MOI 1 and 10 for 48 h. For caspase-3 expression, BMDMs were seeded in 6-well tissue culture plates and were cultured in the presence or absence of L cell-conditioned medium for 16 h. Cells were lysed and boiled in reducing SDS-PAGE sample buffer containing 100 mM Tris, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol and 0.2% bromophenol blue. Lysates were subjected to 4–15% gradient gel electrophoresis (Bio-Rad Laboratories) and transferred to PVDF membrane (Millipore) in 10% methanol, 25 mM Tris and 192 mM glycine, pH 8.3. Anti-Caspase-3 Ab was from Cell Signaling Technology. Rat anti-mouse TREM-2 mAb (clone CI:A3-1; Abcam), anti-mouse phospho-ERK1/2 (clone D13.14.4E, Cell Signaling Technology), anti-mouse TREM-2 (R&D Systems), anti-mouse F4/80 (clone BM8; eBioscience), and anti-mouse Siglec-F (clone E50-2440) were used as primary antibodies and were detected with the appropriate horseradish peroxidase-conjugated secondary antibodies. Chemiluminescence (ECL) system (GE Healthcare). Molecular weight standards were run on each blot to validate that each of the indicated bands corresponded to the appropriate molecular weight.

**Recombinant protein expression and purification.** The mouse TREM-2 Ig domain (amino acid residues 19–136) tagged with 6X His (tTREM-2-his) was expressed, secreted, and purified from mammalian cell culture as previously described (Kober et al., 2014). Precautions were taken to prevent introduction of endotoxin either by using either new, sterile plasticware or by soaking glassware overnight in 0.5 M NaOH. The final purified protein was exchanged in PBS using a microconcentrator (Sartorius). For heat inactivation, tTREM-2-his was boiled at 100°C for 1 h.

**Microscopy.** Tissues were fixed with 10% formalin, embedded in paraffin, cut into 5-μm sections, and adhered to charged slides. Sections were deparaffinized in Citrosoyl (Fisherbrand), hydrated, and in the case of immunofluorescence microscopy, treated with heat-activated antigen unmasking solution (Vector Laboratories). Immunostaining of mouse tissue was performed using anti-MUC5AC mAb (Thermo Fisher Scientific; clone 45M1), anti–Mac-3 mAb (BD; clone M3/84), anti–Caspase-3 mAb (clone 5A1E; Cell Signaling Technology), anti–mouse TREM-2 (R&D Systems), anti–mouse F4/80 (R&D Systems) (clone CLA3-1; Abcam), anti–mouse phospho-ERK1/2 (clone D13.14.4E, Cell Signaling Technology), anti–mouse IL-13 Ab (R&D Systems), and anti-SeV antibody (SPAFAF, Inc.). Antibody binding was visualized using Alexa Fluor 488 or 594 fluorochromes (Invitrogen). Slides were counterstained with DAPI-containing mounting media (Vector Laboratories). For stTREM-2 immunostaining, BMDMs were seeded into 2-well chamber slide (Nalge Nunc International) at 2 × 10^5 cells per well. For surface stTREM-2 immunostaining, BMDMs were incubated with strepT2his (10 µg/ml) for 30 min on ice and then washed three times with chilled PBS and incubated with mouse anti-6X His tag antibody (Abcam; clone His. H8) followed by Alexa Fluor 488–conjugated anti–mouse IgG (Invitrogen) and fixation with 4% PFA. For intracellular stTREM-2 immunostaining, cells were incubated with strepT2his for 30 min at 37°C, washed with PBS, fixed with 4% PFA, and then permeabilized with 0.2% Saponin, 2% BSA in PBS for 30 min before incubation with anti-6X His tag antibody and Alexa Fluor 488–conjugated anti–mouse IgG. All microscopy was performed using an Olympus BX51 microscope and a Retiga-2000R camera system (QImaging).

**Flow cytometry and FACS.** Single-cell lung suspensions were made from minced lung tissue subjected to collagenase (Liberase Blendedzyme III; Roche), hyaluronidase (Sigma-Aldrich), and DNase I (grade II; Roche) digestion for 45 min at 37°C and then treated with ACK buffer to remove red blood cells. Cell percentages were unchanged if lungs were perfused before digestion (suggesting that intravascular cells did not contribute significantly to the analysis), so perfused lungs were used for all experiments. Staining of surface markers was performed using FcR blockage and fluorochrome-conjugated mAb at 4°C. We used the following antibodies: rat anti-mCD11b (clone M1/70; eBioscience), anti–Ly-6C (clone al-21; BD), anti–Ly-6G (clone 1A8; BD), anti–mouse F4/80 (clone BMS; eBioscience), anti–mouse TREM-1 (clone 174031; R&D Systems), anti–mouse TREM-2 (clone 237920; R&D Systems), anti–mouse CD11c (clone HL3; BD), anti–mouse CD45.2 (clone 104; eBioscience), and anti–mouse Siglec-F (clone E50-2440; BD). We used flow cytometry and FACS to identify and purify specific cell populations in mouse lungs using the following gates: tissue monocytes as SSC<sup>hi</sup>Ly-6C<sup>−</sup>Ly-6G<sup>−</sup>Siglec-F<sup>−</sup>F4/80<sup>−</sup>CD11b<sup>−</sup>, interstitial macrophages as SSC<sup>lo</sup>CD11c<sup>−</sup>Ly-6G<sup>−</sup>Siglec-F<sup>−</sup>F4/80<sup>−</sup>CD11b<sup>−</sup>, alveolar macrophages as SSC<sup>hi</sup>CD11c<sup>−</sup>Ly-6G<sup>−</sup>Siglec-F<sup>−</sup>F4/80<sup>−</sup>CD11b<sup>−</sup>, and neutrophil gate as SSC<sup>hi</sup>Ly-6G<sup>−</sup> (Fig. S1). Analysis was performed using MoFlo (DAKO-Cytomation) and FlowJo software (Tree Star). Cells were counted using flow cytometry and fluorescent counting beads (CountBright; Life Technologies).

**Cell culture.** Bone marrow cells were flushed from femurs and differentiated in 150 × 15 mm Petri dishes in DMEM medium containing 0.29 g/liter l-glutamine, 1 mM sodium pyruvate, 10% FBS, and 30% L929 fibroblast-conditioned medium as a source of CSF-1. At day 6 or indicated times, BMDMs were resuspended by incubation in 1 mM EDTA in PBS for 10 min on ice, washed twice with PBS and plated into tissue culture plates. In some experiments, BMDMs were incubated with poly(C) (Sigma-Aldrich) or mouse IL-13 (R&D Systems). For cell viability assays, BMDMs were seeded into 96-well tissue culture plates at 5 × 10^4 cells per well and were assessed for viability using the Cell Proliferation kit (XTT) from Roche. For cell proliferation assays, BMDMs were seeded into 6-well tissue culture plates, labeled for 18 h with BrdU (10 μM), and then assessed using flow cytometry with the FITC BrdU Flow kit (BD).

**Apoptosis assays.** For propidium iodide (PI) assay, BMDMs were cultured in L cell–conditioned medium for 5 d and deprived of CSF-1 for 1 d to induce apoptosis (Otero et al., 2009). Cells were detached using EDTA (1 mM) in PBS, washed, fixed with 70% ethanol, and incubated with PI (50 µg/ml) and R-Nase (40 µg/ml) for 1 h. Samples were analyzed for DNA content using a FACScan (BD). For Annexin V assay, BMDMs were differentiated in L cell–conditioned medium for 5 d, detached with EDTA, seeded onto the poly-D-Lysine-coated coverslips (BD) placed in a 24-well plate, and cultured in medium without CSF-1 for 1 d. PI and Annexin V immunostaining was performed according to the manufacturer’s protocol (Invitrogen). Annexin V–positive, PI-negative staining cells were quantified using microscopy, and values were expressed as the percentage of apoptotic cells (Annexin V positive, PI negative) relative to the total number of cells in 6 randomly selected fields of the two wells.

**Monocyte influx.** For analysis of monocyte influx into the lung, BMMs were purified from CD45.1 WT and C57BL/6J WT mice and were infected with SeV at MOI 1 and 10. Cell supernatant and lysate samples, BMDM (2 × 10^6 cells in 2 ml of complete DMEM containing 15% L cell–conditioned medium) were seeded into 12-well tissue plates, treated with or without IL-13 (20 ng/ml) or SeV (MOI 1 and 10). Cell supernatants were collected, and cells were washed twice with chilled PBS and then lysed in radio-immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) containing protease inhibitors. Levels of CSF-1 were determined using a DuoSet ELISA kit (R&D Systems) and levels of cellular TREM-2 and stTREM-2 with a TREM-2 ELISA kit (Antibodies-online.com).

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Statistical analysis. For experiments in mice and cell culture, all experiments were repeated a minimum of three times, and representative values are presented. PCR data were presented as mean ± SEM and was analyzed using an unpaired Student’s t-test. If variances were unequal, Welch’s correction was applied. For all analyses, \( P < 0.05 \) were considered significant. For SeV-epithelial cell co-cultures, the IL-33-producing epithelial progenitor cells in chronic obstructive lung disease. J. Clin. Invest. 123:3967−3982. doi.org/10.1172/JCI65770
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