Slamf7 is dispensable in mouse models of acute lung injury

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To the Editor:

During the course of acute respiratory distress syndrome (ARDS), inflammatory cells contribute to destruction of the alveoli and lung parenchyma, leading to respiratory failure. Airspace macrophages play key roles in the pathogenesis of acute lung injury (ALI) and ARDS by producing inflammatory cytokines, recruiting neutrophils, and releasing cytotoxic and apoptotic factors that directly damage the lung epithelium [1]. Therefore, manipulating macrophage function during ALI may be useful for treating or preventing ARDS.

Our group has previously shown that monocytes that are recruited to the lungs during acute inflammation mature into recruited airspace macrophages (RecAMs) that have proinflammatory and profibrotic transcriptional profiles [2, 3]. In an effort to understand the factors that regulate RecAM functions, we interrogated our RNA sequencing dataset for novel candidate genes that are differentially expressed between resident airspace macrophages (ResAMs) and RecAMs [4]. We discovered that Slamf7 was highly expressed by RecAMs but absent in ResAMs (figure 1a). The role of Slamf7 expression by RecAMs in ALI is unknown. Importantly, others have reported a higher proportion of Slamf7-expressing macrophages in the bronchoalveolar lavage (BAL) of patients with more severe COVID-19 compared to those with less severe disease [5]. In contrast, Slamf7 was found to suppress inflammation in mouse models of sepsis [6]. We hypothesised that *Slamf7* deletion would ameliorate inflammation in murine models of direct ALI.

The signalling lymphocytic activation molecule (SLAM) family of proteins are cell surface glycoproteins found on most immune cells, and are known to have roles in cell–cell adhesion and immune regulation. In general, SLAM family members function as self-ligands that signal through immunoreceptor tyrosine switch motifs to regulate immune responses [7]. In macrophages, SLAM family member 7 (Slamf7) expression is upregulated with *in vitro* exposure to inflammatory stimuli such as lipopolysaccharide (LPS) and flagellin [8–11].

Slamf7 knockout (KO) C57BL/6 mice were generously provided by the Veillette laboratory and do not have obvious defects in development or any overt phenotype [12, 13]. The mice were bred at our facility. Male and female mice aged 10–16 weeks were used for all experiments. KO mice were compared to wildtype (WT) littermates. Groups were age- and sex-matched in all experiments. Mice were treated with 80 µg *Escherichia coli* LPS (representing Gram-negative infection), 2.5 plaque-forming units of PR8 influenza (representing viral infection) or 1.5 $U \cdot kg^{-1}$ bleomycin (representing oxidant-induced ALI with transient fibrosis) in 50 µL PBS by intratracheal instillation using a modified gel-loading tip under general anaesthesia with isoflurane. BAL was collected by lavage of the lungs with 1 mL PBS with 5 mM EDTA five times. BAL was analysed by flow cytometry using markers for ResAMs ($SiglecF^+$), RecAMs ($SiglecF^-$) and neutrophils ($Ly6G^+$) to compare the inflammatory cell milieu. Albumin, tumour necrosis factor (TNF)-α and interleukin-6 were measured in the supernatant from the first 1 mL BAL by ELISA. For the bleomycin injury model, lung static compliance (SCIREQ flexiVent) and collagen content (hydroxyproline) were measured at day 21.

Flow cytometry confirmed that Slamf7 protein was highly expressed on RecAMs, and absent on ResAMs, in WT mice (figure 1a). Because Slamf7 is thought to signal *via* homotypic interactions, we analysed BAL for other cell types expressing Slamf7 and found that RecAMs are the predominant $Slamf7^+$ population, with natural killer (NK) cells and a small fraction of B- and T-cells also expressing Slamf7 (data not shown). Flow cytometry also confirmed that Slamf7 was absent from RecAMs from *Slamf7* KO mice (figure 1a).

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Slamf7 is expressed by monocyte-derived macrophages recruited to the lungs during injury. Whole-body and macrophage-specific knockouts of *Slamf7* had no effect on the degree of inflammation in three mouse models of acute lung injury. https://bit.ly/3KgTJg1

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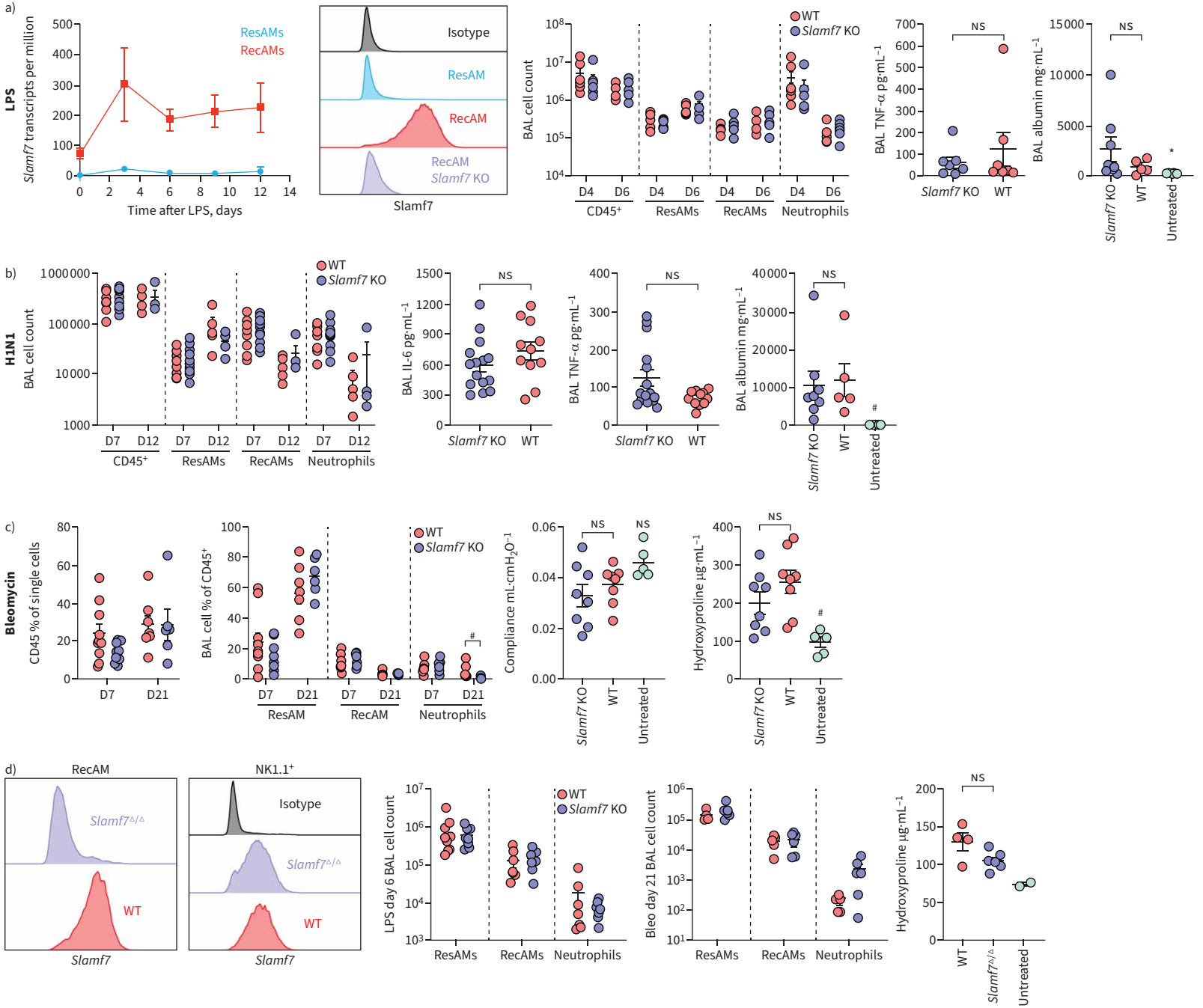

FIGURE 1 Slamf7 is expressed on recruited airspace macrophages (RecAMs), but not resident airspace macrophages (ResAMs), and its deletion has no effect on inflammation in three murine models of acute lung injury. a) *Slamf7* transcripts per million from bulk RNA sequencing of sorted ResAMs and RecAMs from bronchoalveolar lavage (BAL) of lipopolysaccharide (LPS)-treated mice. Histogram of Slamf7 expression on RecAMs and ResAMs from wildtype (WT) mice and RecAMs from *Slamf7* knockout (KO) mice analysed 6 days after LPS. Slamf7 protein expression was detected by flow cytometry. ResAMs are identified by expression of SiglecF, while RecAMs are SiglecF^{-}. Macrophages were gated as CD45^{+}, Ly6G^{-}, CD64^{+} and CD88^{+}. Leukocyte numbers, tumour necrosis factor (TNF)-α and albumin in BAL from WT and *Slamf7* KO mice following administration of intratracheal LPS (80 µg). TNF-α and albumin measured from BAL of *Slamf7* KO and WT mice 4 days after intratracheal (*i.t.*) LPS. b) Leukocyte numbers and BAL interleukin (IL)-6, TNF-α and albumin measured in *Slamf7* KO and WT mice at day (D)7 and D12 after *i.t.* PR8 influenza A (H1N1). c) Percentages of cell types in BAL were compared at D7 and D21 after *i.t.* bleomycin. Lung static compliance was measured *via* FlexiVent at D21 after bleomycin in *Slamf7* KO and WT mice, with untreated WT mice as controls. Hydroxyproline content of the right lung was measured at D21 after bleomycin, with untreated WT mice as controls. d) Slamf7 protein is not detected on RecAMs from *Slamf7*$^{\Delta/\Delta}$ mice by flow cytometry but is detected on NK1.1^{+} lymphocytes in both *Slamf7*$^{\Delta/\Delta}$ and WT control littermates at D6 after LPS. Numbers of neutrophils, SiglecF^{+} ResAMs and SiglecF^{-} RecAMs in BAL were determined by flow cytometry, and were not different between *Slamf7*$^{\Delta/\Delta}$ mice and WT littermates at D21 after bleomycin, nor was whole-lung collagen content measured by hydroxyproline assay. All statistical comparisons between *Slamf7* KO or *Slamf7*$^{\Delta/\Delta}$ and WT mice were not significant (p>0.05, t-test or Mann–Whitney U-test) except where indicated. For BAL albumin, compliance and hydroxyproline assays, *Slamf7* KO and WT treated mice were significantly different from untreated mice as indicated. *: p<0.05, Kruskal–Wallis test; #: p<0.005, Kruskal–Wallis test. NS: nonsignificant.

Analysis of BAL cell populations 1 (not shown), 4 and 6 days after LPS revealed no differences in total CD45^{+} cells, neutrophils, ResAMs or RecAMs between *Slamf7* KO mice and WT littermates, and no difference in BAL TNF-α or albumin (figure 1a). Similarly, no differences were observed in these parameters at 7 or 12 days after PR8 influenza infection (figure 1b). Finally, in the bleomycin model of ALI with transient fibrosis, there were no differences in the relative abundance of cell types in the BAL between *Slamf7* KO and WT littermates at 7 or 21 days after bleomycin, with the exception of decreased neutrophils in *Slamf7* KO mice at day 21 compared to WT mice. Furthermore, there were no differences in static compliance or hydroxyproline content, as indicators of the degree of fibrosis, at day 21 (figure 1c). There were no differences in B-cell, CD4^{+} or CD8^{+} T-cell, or NK cell numbers in any of the injury models (data not shown).

To account for possible opposing effects of Slamf7 in other immune cells, we generated *Slamf7*$^{fl/fl}$ mice using CRISPR-mediated insertion of *loxP* sites flanking *Slamf7* exon 2. These mice were subsequently crossed to *LysM*^{Cre} mice (Jackson Labs) to generate *LysM*^{Cre}-*Slamf7*$^{fl/fl}$ mice (designated as *Slamf7*$^{\Delta/\Delta}$ hereafter and in figure 1). Since *Slamf7* is not expressed by any LysM-expressing structural cells, ResAMs, neutrophils or most dendritic cells, *Slamf7*$^{\Delta/\Delta}$ mice are essentially a RecAM-specific KO. *Slamf7*$^{\Delta/\Delta}$ mice have *Slamf7* conditionally deleted in myeloid cells (figure 1d). There were no differences in BAL neutrophil, ResAM or RecAM numbers in LPS- or bleomycin-treated *Slamf7*$^{\Delta/\Delta}$ compared to littermate controls (figure 1d).

Taken as a whole, our data show that Slamf7-expressing RecAMs were abundant in the airspace in multiple murine models of direct lung injury. Contrary to our initial hypothesis, we discovered no differences in BAL immune cell numbers or inflammatory cytokine levels between WT and *Slamf7* KO mice in sterile (LPS) or infectious (H1N1) lung injury models. We similarly found no differences in BAL immune cell numbers, static compliance or collagen content between WT and *Slamf7* KO mice in the bleomycin model of ALI with transient lung fibrosis. One explanation for the lack of phenotype in *Slamf7* KO mice is the possibility of functional redundancy between Slamf7 and other SLAM family members. Additionally, Slamf7 has been demonstrated to mediate both pro- and anti-inflammatory effects in different *in vitro* and *in vivo* models, and in different immune cell types [5, 10, 14]. For instance, Slamf7 is known to activate NK cells and has been shown to induce exhaustion in T-cells [7, 15]. To address the possibility of opposing effects from other immune cells, we generated a myeloid-specific knockout of *Slamf7*. Again, there were no differences in BAL cell counts or collagen content in LPS- or bleomycin-treated *Slamf7*$^{\Delta/\Delta}$ mice compared to WT littermate controls.

Our data are in contrast to findings published by Wu *et al.* [6], who found that macrophage Slamf7 was protective in mouse models of sepsis and reduced sepsis-induced lung injury. The reason for this discrepancy is unknown; however, a key difference is direct injury in our models compared to indirect lung injury in the sepsis models. Peripheral monocytes upregulate Slamf7 upon exposure to LPS, so it is possible that the intravenous treatment route used by Wu *et al.* [6] results in additional Slamf7-mediated effects on intravascular monocytes. Moreover, the sepsis models were lethal, with mice succumbing to shock in a matter of hours to days, whereas our models were sublethal. Additionally, we found no association of Slamf7 expression with more severe disease as described in the studies of human COVID-19

[5]. It is possible that Slamf7 mediates different effects in mice and humans, and we did not investigate this possibility. Collectively, Slamf7 may have important functions in other tissues or other injury models, but global and macrophage-specific knockouts of *Slamf7* had no effect on the severity of inflammation in three different murine models of direct ALI.

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