S100A8/A9 and S100A9 reduce acute lung injury

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S100A8 and S100A9 are myeloid cell-derived proteins that are elevated in several types of inflammatory lung disorders. Pro- and anti-inflammatory properties are reported and these proteins are proposed to activate TLR4. S100A8 and S100A9 can function separately, likely through distinct receptors but a systematic comparison of their effects in vivo are limited. Here we assess inflammation in murine lung following S100A9 and S100A8/A9 inhalation. Unlike S100A8, S100A9 promoted mild neutrophil and lymphocyte influx, possibly mediated in part, by increased mast cell degranulation and selective upregulation of some chemokine genes, particularly CXCL-10. S100 proteins did not significantly induce proinflammatory mediators including TNF-α, interleukin-1β (IL-1β), IL-6 or serum amyloid A3 (SAA3). In contrast to S100A8, neither preparation induced S100A8 or IL-10 mRNA/protein in airway epithelial cells, or in tracheal epithelial cells in vitro. Like S100A8, S100A9 and S100A8/A9 reduced neutrophil influx in acute lung injury provoked by lipopolysaccharide (LPS) challenge but were somewhat less inhibitory, possibly because of differential effects on expression of some chemokines, IL-1β, SAA3 and IL-10. Novel common pathways including increased induction of an NAD+-dependent protein deacetylase sirtuin-1 that may reduce NF-κB signalling, and increased STAT3 activation may reduce LPS activation. Results suggest a role for these proteins in normal homeostasis and protective mechanisms in the lung.

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suppressor cells but functions of S100A8 in this context are unclear.17

Extracellularly, S100A8 and S100A9 are traditionally considered proinflammatory although functions in lung inflammation are controversial. Tumour necrosis factor-α (TNF-α) induction was proposed to initiate inflammation following intratracheal S100A9 delivery to normal mice although intranasal delivery using an adenoviral vector failed to do so.18 The latter predominantly promoted a macrophage influx 8 days post-challenge in a response independent of the two putative receptors: TLR4 and RAGE.19 More recently, unexpected protective effects of S100A9 and S100A8/A920,21 were reported, and in humans with sepsis, delayed overexpression of S100A9 was associated with occurrence of secondary hospital-acquired infections, again implying protective effects.22 Consistent with this, S100A9 maintains circulating neutrophil numbers and recruitment to Streptococcus pneumoniae-infected murine lungs, principally by regulating G-CSF production23 and S100A9-deficiency is associated with increased cytokine levels and aggravated lung pathology in mice infected with S. aureus.20

Our studies indicate a role for S100A8 in immunoprotection and/or resolution of inflammation.10,11 Unlike S100A8, S100A9 is not induced in murine macrophages by TLR agonists;2 S100A8, induction is IL-10-dependent, regulated in a manner similar to the IL-10 gene and enhanced by corticosteroids, responses uncharacteristic of TLR agonists. Importantly, S100A8 inhibition inhibited ALI in a manner similar to dexamethasone, and may contribute to immunosuppressive functions of corticosteroids.11 Together with the differences in their structure, potential post-translational modifications and putative receptors, we proposed that S100A9 may promote different responses to S100A8 in murine lung. Here we present a systematic comparative study of the functional effects of S100A9 and S100A8/A9 inhalation in normal lung and propose distinct modulatory roles.

Unlike S100A8,11 S100A9 and S100A8/A9 inhalation directly promoted mild neutrophil influx, possibly mediated in part by increased mast cell (MC) degranulation and differential upregulation of particular chemokine genes. Unlike LPS, low or high dose intranasal S100A9 did not induce significant amounts of TNF-α or CCL-2. CXCL-10 was only elevated by S100A9. Neutrophil influx in response to LPS inhalation was suppressed by S100A9 and S100A8/A9 although there were differential effects on expression of some chemokine genes, IL-1β and the acute phase reactant serum amyloid A3 (SAA3), which promotes lung neutrophilia,23 only S100A8 significantly suppressed SAA3 in ALI. In marked contrast to S100A8, neither S100A9 nor the complex induced IL-10 in the airways, or in tracheal epithelial cells in vitro. Characterisation of the receptors for these S100 proteins in lung would likely reveal novel mechanisms that modulate complementary immunoregulatory roles of S100A8, S100A9 and S100A8/A9.

RESULTS

The model used here was one commonly employed to study LPS-induced lung injury. Because they are reported TLR4 agonists, we first examined effects of inhalation of S100A9 and S100A8/A9 in lungs from naïve mice. When doses of S100A9 were tested, 10 μg was chosen for studies over a time course (1–12 h), so that we could capture mediator production at early and later stages, and could be compared with results published for a similar dose of S100A8.11 The ability of these proteins to modulate inflammation induced by LPS challenge was tested by administering 10 μg intranasally 2 h before LPS inhalation, and lungs harvested 4 h later, a time point when LPS-induced genes are highly expressed.

S100A9 and S100A8/A9 promote neutrophil and lymphocyte recruitment in naïve lung

Total leucocyte numbers in BALF from control (Hanks balanced salt solution (HBSS)-treated) mice were constant over 20 h. Lymphocytes (~0.5–1% total leucocytes) were elevated 2–3.3-fold by S100A9 at 1, 4, 6 and 12 h post-inhalation (Figure 1a) and at 1 and 4 h were similar to lymphocyte numbers elicited by LPS alone (2.4 ± 0.5 and 3.4 ± 0.4 × 10^6; n = 7, respectively). Neutrophils were rare in bronchial lumen fluid (BALF) from HBSS-treated mice. In comparison, significant neutrophil infiltration was evident 4 h post S100A9-inhalation even though numbers were low (2.7–3.1 × 10^6) and decreased gradually over 12 h, with a second peak at 20 h (Figure 1b). In contrast, BALF harvested 4 h post LPS-treatment contained some 10–15-fold more neutrophils (55.3 ± 11.0 × 10^6; n = 7). S100A8/A9 promoted similar lymphocyte and neutrophil recruitment although numbers were somewhat less than seen with S100A9 6–20 h post-inhalation (Figures 1a and b). Similar to S100A8,11 infiltration of S100-positive myeloid cells within lung tissue was negligible 1 or 4 h post S100A9 inhalation whereas S100A8/A9 caused a mild influx of S100A9-positive/S100A8-negative myeloid cells within the extravascular compartment, and S100A9 deposits were obvious on the extracellular matrix (Figure 1c).

Expression of genes that may modulate leucocyte recruitment in lung post S100 or LPS administration, relative to HBSS control, are shown in Supplementary Tables 1 and 2 respectively. LPS significantly increased most chemokine genes including CCL-2 (27-fold), CXCL-1 (34-fold), CXCL-2 (90-fold), CXCL-9 (23.6-fold) and CXCL-10 (231-fold). In contrast, S100A9 stimulated low but significant increases in some chemokine genes (CCL-3, 4, CXCL-1, 2, 9 and 10) within an hour; except for CXCL-10, messenger RNAs (mRNAs) declined, whereas CXCL-10 mRNA was some 30.9-fold above controls at 12 h. CXCL-10 concentrations in BALF harvested 4 h post S100A9 inhalation increased from 30.8 ± 9.3 pg ml^-1 at baseline, to 102.7 ± 7.0 pg ml^-1 (P < 0.001; Figure 1d) but receded thereafter. In contrast, and in keeping with mRNA expression, CXCL-10 concentrations in BALF from mice treated with S100A8/A9 were not significantly different from controls. Similar to S100A9, S100A8/A9 increased CXCL-1 and CXCL-2 mRNAs 6–10-fold after 1 h. Unlike LPS, CXCL-2 mRNA was not significantly elevated.

SAA is proinflammatory24 and promotes neutrophilic infiltration in the lung.25 In marked contrast to its very strong induction by LPS alone (Supplementary Table 2), endogenous SAA3 mRNA was reduced by both S100 preparations (Supplementary Table 1) and the negative response to S100A9 was sustained over 12 h. ICAM-1 mediates neutrophil adhesion; unlike S100A8/A9, S100A9 significantly increased ICAM-1 mRNA 12 h post inhalation (24.6-fold above control), and may facilitate the later elevated neutrophil influx seen at 20 h (Figure 1b). Matrix metalloproteases (MMP) influence leucocyte transmigration and are modulated by S100A8/A9 in some cells.22 Supplementary Table 1 shows that S100A9 increased MMP-2 mRNA 2.8–3.5-fold over 1–12 h whereas induction by S100A8/A9 was low, and delayed (4–6 h). Induction of MMP-9 mRNA by S100A9 (5.4-fold increase) and S100A8/A9 (3.7-fold) was rapid but decreased 1 h post inhalation. However immunohistochemistry failed to detect MMP-9 in lung specimens from any sample set harvested over 1–20 h.

Differential effects of S100s on pro- and anti-inflammatory genes in naïve lung

S100A9 and S100A8/A9 marginally increased TNF-α mRNA 1 h post challenge (Supplementary Table 1); no significant increases were found at later time points. BALF harvested 1–20 h post inhalation of
the S100 preparations did not contain significantly elevated TNF-α above concentrations in control BALF (3.2 ± 0.2 pg ml\(^{-1}\) (lower limits of assay); \(n=4\) group, \(P<0.05\)). Moreover TNF-α concentrations in BALF from mice treated with 1, 10 or 25 μg S100A9 were similar, and not significantly greater than control concentrations (Supplementary Table 3). This contrasts markedly with the increased gene expression (Supplementary Table 2) and high amounts of TNF-α induced by LPS (684.8 ± 124.5 pg ml\(^{-1}\); Table 1).

Changes in IL-1β mRNA were also slight; BALF from mice treated with S100A8/A9 or S100A9 harvested at 12 h contained 7.7 ± 1.4 and 8.0 ± 1.0 pg ml\(^{-1}\) respectively, some threefold more than in BALF from mice treated with HBSS (2.3 ± 1.1 pg ml\(^{-1}\); \(n=4\), \(P<0.05\)). Interestingly, at 1 h post-S100A9 inhalation, basal expression of the IL-1 receptor antagonist (IL-1RA) gene was significantly reduced by 50-fold; S100A8/A9 caused a 6.7-fold decrease that was only obvious in lung samples harvested at 4–6 h (Supplementary Table 1). Only S100A8/A9 induced low and transient IL-6 mRNA expression 1 h post-inhalation but amounts in BALF were similar to controls. In our hands, we detected little direct induction of these cytokines following \textit{in vitro} incubation of tracheal epithelial cells, the human epithelial cell line A549, or murine elicited macrophages, murine/human monocytes or monocyte-derived macrophages, or various monocyte/macrophage cell lines stimulated using the appropriate murine or human S100 proteins over a dose range (0–10 μg ml\(^{-1}\) for most experiments).

S100A9 inhalation increased IFN-γ mRNA over 12 h by 17.3-fold whereas S100A8/A9 caused a transient increase 4–6 h post inhalation (Supplementary Table 1). However quantities in BALF from S100A9-treated mice were very low (2.3 ± 0.5 pg ml\(^{-1}\)) and at other time points, below the level of detection (1.95 pg ml\(^{-1}\)).

We next examined IL-10 mRNA in lung tissue over 1–20 h. Compared to the 1183-fold increase induced at 12 h by S100A8,\(^{11}\) S100A8/A9 or S100A9 did not significantly alter basal IL-10 mRNA over the time course (Supplementary Table 1), and different S100A9

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**Figure 1** S100A9 promotes neutrophil recruitment and CXCL-10 production. Total numbers of (a) lymphocytes and (b) neutrophils in BALF from control (HBSS), S100A9 or S100A8/A9-treated mice (both 10 μg; harvested 1, 4, 6, 12 and 20 h post inhalation). (c) Anti-S100A8 and -S100A9 immunoreactivities of lung sections from mice harvested 1, 4 or 12 h post S100A9 or S100A8/A9 inhalation. Sections are representative of 4 mice per group; scale bars, 50 μm. S100A9-positive airway lining cells are indicated with arrows. (d) CXCL-10 in BALF from mice treated with S100A9 or S100A8/A9 (1, 4, 6 and 12 h post inhalation). Data are means ± s.e.m., \(n=4\) mice/group; *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\), ****\(P<0.0001\) compared with HBSS-treated mice.

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**Table 1** S100A9 or S100A8/A9 effects on SAA3 and mediator concentrations in BALF induced by LPS

|        | HBSS+PBS | HBSS+LPS | S100A9+LPS | S100A8/A9+LPS |
|--------|----------|----------|------------|---------------|
| SAA3   | 13.6 ± 0.8** | 31.9 ± 3.0 | 27.2 ± 5.2  | 34.6 ± 3.2    |
| TNF-α  | 26.6 ± 8.1*** | 684.8 ± 124.5 | 164.4 ± 105.2** | 159.3 ± 46.3** |
| IL-1β  | 6.6 ± 2.5   | 25.2 ± 3.9  | 13.6 ± 2.7   | 38.4 ± 7.3    |
| IL-6   | 2.0 ± 1.3** | 88.7 ± 22.0 | 5.1 ± 4.4**  | 36.6 ± 10.6   |
| CCL-2  | 3.6 ± 1.6*  | 23.5 ± 7.7  | 6.0 ± 1.2    | 11.4 ± 1.1    |
| CXCL-10| 45.2 ± 19.5 | 200.2 ± 77.5| 9.9 ± 3.7*   | 97.1 ± 16.6   |

**Abbreviations:** HBSS, Hanks balanced salt solution; IL, interleukin; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; SAA3, serum amyloid A3; TNF, tumour necrosis factor.

| Mean ± SEM | p-value |
|------------|---------|
|            |         |
|            |         |
|            |         |
|            |         |

S100A8/A9 and S100A9 reduce acute lung injury in response to LPS

Expression of mRNAs encoding inflammatory genes was generally low in lungs from mice harvested 1 h post LPS inhalation (for example, CCL-2: 4.3-fold, CXCL-10: 5.8-fold above control), compared to the high expression, particularly of chemokine genes, at 4 h (CCL-2: 27-fold, CXCL-10: 231-fold above control values), so this time point was chosen for most analyses. Supplementary Table 2 and the heat map shown in Figure 3a compare chemokine and cytokine/proinflammatory gene expression in S100 or HBSS (control)-pretreated lungs challenged 2 h later with LPS. The volcano plot (Figure 3b) shows that several cytokine and chemokine genes were significantly reduced by > 2.5-fold (P = 0.05) in LPS-challenged lungs from mice pretreated with S100A9 compared to control (HBSS); no genes were significantly upregulated by S100A9. S100A9 pretreatment reduced TNF-α mRNA expression in lungs of LPS-challenged mice (Figure 3b) from 14.2-fold to 3-fold above baseline. TNF-α in BALF was also significantly less (164.4 ± 105.2 pg ml⁻¹ compared to 684.8 ± 124.5 pg ml⁻¹ with LPS alone; Table 1). Moreover, inhalation of 1 or 10 μg S100A9 suppressed TNF-α induction by LPS to the same extent, suggesting high potency; the high dose (25 μg) was also significantly suppressive (Supplementary Table 4). S100A8/A9 similarly reduced TNF-α mRNA and concentrations in BALF. IL-6 mRNA was some fivefold less in lungs of mice pretreated with S100A9, and twofold less with S100A8/A9 compared to LPS alone (Supplementary Table 2). These changes were reflected by IL-6 concentrations in BALF (5.1 ± 4.4 and 36.6 ± 10.6 pg ml⁻¹ respectively, compared to 88.7 ± 22 pg ml⁻¹ with LPS alone; Table 1). IL-1β mRNA induction by LPS was reduced from ~ 8 to ~ 4-fold by S100A9 and IL-1β concentrations in BALF were ~ 50% less than in BALF from mice challenged with LPS alone; S100A8/A9 had no significant effect (Supplementary Table 2 and Table 1). S100A9 inhalation also significantly reduced mRNA induction of IL-1RA, CSF2, and MMP13 by LPS whereas S100A9 had little effect (Figure 3c). The low amounts of IL-10 induced by LPS were reduced to approximately the same extent by 1.25 μg S100A9 (Supplementary Table 4).

Most chemokine genes induced by LPS were markedly suppressed by S100A9; S100A8/A9 was somewhat less effective (Figure 3 and Supplementary Table 2). For example, the 231-fold upregulation of CXCL-10 mRNA by LPS remained at baseline in lungs from mice pretreated with S100A9, but were fourfold above baseline values with S100A8/A9. Similarly, CCL-2 mRNA increased ~ 27-fold following LPS inhalation; S100A9 pretreatment suppressed induction to almost background whereas in lungs from S100A8/A9-treated mice, CCL-2 mRNA expression was only 2.4-fold less than with LPS alone. CCL-2 and CXCL-10 concentrations in BALF were less in S100A9-, than in S100A8/A9-pretreated mice, compared to LPS alone (Table 1); 1, 10 or 25 μg S100A9 were all suppressive (Supplementary Table 4). We next tested whether tracheal epithelial cells were S100-responsive in vitro and included S100A9 as this had not been tested. No S100 preparation directly induced significant chemokine gene expression whereas LPS increased CCL-2 and CXCL-10 mRNAs some 6- and 36-fold, respectively (Figures 4a and b). Although S100A9 was somewhat suppressive, S100A8/A9 and S100A8 significantly reduced CCL-2 mRNA induction by LPS to about the same extent. CXCL-10 mRNAs were reduced by all preparations but only S100A8 was significantly suppressive (by 69%); Figure 4b).

The high SAA3 mRNA induced by intranasal LPS (419-fold above basal expression) was reduced to 85-fold by S100A9 whereas S100A8/A9 inhalation was ineffective (Supplementary Table 2). In keeping with this, SAA3 was significantly above...
control concentrations in BALF from LPS-challenged mice, but concentrations were not significantly reduced by S100 pretreatment (Table 1). SAA3 is produced by airway epithelial cells and we sought to validate its regulation by S100 proteins using tracheal epithelial cells in vitro. Interestingly, S100A8/A9 significantly increased SAA3 mRNA above controls (P<0.05) but S100A8 and S100A9 had little effect. S100A9 and S100A8/A9 weakly suppressed SAA3 mRNA induction by LPS in vitro whereas in keeping with our earlier findings in vivo, S100A8 was significantly inhibitory (Figure 4c).

Signalling pathways contributing to suppression of ALI by S100A9 and S100A8/A9
The MAPK pathway mediates signalling in response to LPS. LPS inhalation reduced MAPK phosphatase (MKP, a key regulator of this pathway) gene expression in lung whereas S100A9 and S100A8/A9 pretreatment tended to increase mRNA (Supplementary Table 2). Western blotting confirmed decreased endogenous MKP-1 concentrations in lung lysates following LPS inhalation. Unexpectedly, the S100 preparations reduced MKP-1 below levels found in LPS or control samples (Figure 5a). When phosphorylation of p38 and Erk1/2 was...
compared, no significant changes in P-p38 were obvious (not shown); S100A9 and S100A8/A9 pretreatments increased p-Erk1/2 above amounts found in lungs from mice treated with LPS alone (Figure 5a). p-MEK1/2 levels were similar with all treatments (Figure 5a). Because changes may have occurred earlier than 4 h post LPS challenge, lysates of lungs harvested 1 h post LPS challenge were also examined, but no significant differences in MKP-1, or in phosphorylation levels of the MAPKs were found between groups (Figure 5b).

Figure 3 Comparison of S100A9 and S100A8/A9 pretreatments on genes induced by LPS. (a) Clustergram of mRNA expression of genes in lung tissue from mice after administration of HBSS+PBS (control), HBSS+LPS, S100A9+LPS or S100A8/A9+LPS. Hierarchical clustering of the entire data set was performed to provide a heat map with dendograms indicating co-regulated genes across groups; individual samples are shown, to validate variations in reactivity within groups. (b and c) Volcano plot of mRNAs that were differentially regulated, with changes > 2.5-fold, and $P<0.05$ (blue line); plots arrange fold differences along the x-axis and statistical significance along the y-axis. Results show mRNAs differentially regulated in lungs from (b) HBSS-pretreated or (c) S100A8/A9-pretreated mice, then challenged with LPS, compared to S100A9-pretreated then LPS-challenged mice; $n=5$ mice per group.
NF-κB mediates gene induction by LPS; S100A9 pretreatment did not significantly alter NF-κB (p50) mRNA expression whereas S100A8/A9 significantly increased expression by LPS (Figure 6a). Formation of p50/p50 homodimer can reduce LPS activation. Western blotting confirmed significantly decreased p50 levels in LPS-treated lung. S100A9 pretreatment prevented this decrease, and p50 levels were similar to those in control extracts; S100A8/A9 exhibited a similar trend and both preparations elevated p105 levels compared to LPS alone (Figure 6b). Although S100A9 somewhat reduced IkBα levels 1 h post LPS challenge, these were significantly increased after 4 h (Figure 6c). Silent information regulator T1 (SIRT1) can also antagonise NF-κB signalling to suppress inflammation.29 As expected, SIRT1 in lung extracts was significantly decreased by LPS inhalation. S100A9 pretreatment prevented this reduction and S100A8/A9 caused a similar trend (Figure 6d).
Suppressor of cytokine signalling 3 modulates ALI in response to LPS and can negatively regulate signalling via STAT3. Here we confirm elevated SOCS3 mRNA in lungs from mice challenged with LPS, but unexpectedly, S100 pretreatment significantly restrained SOCS3 induction, which was similar to that in controls (Figures 3b and 6c). On the other hand, S100A9 and S100A8/A9 pretreatment significantly increased STAT3 mRNA above that expressed in lungs from mice only given LPS (Figure 6f) and Western blotting indicated somewhat higher phosphorylated STAT3 in samples from mice pretreated with the S100s (Figure 6g), suggesting activation.

Figure 6 Pathways contributing to suppression of ALI by S100A9 and S100A8/A9. (a, e and f) Relative mRNA levels of (a) NF-κB, (e) SOCS3 and (f) STAT3 in lung tissue from mice treated with S100A9 or S100A8/A9 for 2 h, then LPS for 4 h. Averages of HPRT, β-actin and GAPDH were used as internal housekeeping genes (shown as HKG). Data are means±s.e.m., n=5 mice per group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 versus HBSS+LPS group. (b–d) Western blots and quantitative signal intensities of densitometry of bands corresponding to (b) NF-κB, (c) IκBα, (d) SIRT1 and (g) p-STAT3 in lung lysates from mice treated with S100A9 or S100A8/A9 for 2 h and then LPS for 1 h (IκBα) or 4 h (NF-κB, IκBα, SIRT1 and p-STAT3). Representative blots are shown; densitometry shown as means±s.e.m. of bands relative to β-actin; n=5 individual lung lysates/group in two independent analyses. *P<0.05, **P<0.01 versus HBSS+LPS group.
DISCUSSION
The lung is constantly challenged with TLR ligands that trigger robust reactions important in host defence. To limit tissue damage, the epithelial innate and inflammatory response to pathogens is controlled by numerous regulatory mechanisms, including hypo-responsiveness to TLR activation. S100A8 and S100A8/A9 are reported TLR4 and RAGE ligands, although other receptors are implicated in S100A9 function. Proinflammatory properties of S100A9 and S100A8/A9, either directly, or in combination with LPS are reported. S100A9 and S100A8/A9 can exhibit distinct extracellular functions, and here we show both to be only weakly proinflammatory compared to the intense induction of the TNF-α, IL-1β, IL-6, SAA3 and chemokine genes typical of LPS (Supplementary Tables 1 and 2). In lungs harvested 1 h post S100A9 or S100A8/A9 inhalation, TNF-α mRNA increased ~2-fold; IL-1β was unaffected; IL-6 mRNA only increased (some 4.5-fold) with S100A8/A9. These increases rapidly declined and cytokine concentrations in BALF did not reflect a robust TLR4-mediated response (Supplementary Tables 1 and 3). Different functions of high and low doses of some 100 proteins are reported, but we found no significant differences in cytokine concentrations in BALF following inhalation of 1, 10 or 25 μg S100A9 either with or without LPS challenge (Supplementary Tables 3 and 4). Here we used 10 μg S100A9 to capture a comprehensive picture of inflammatory gene expression in naïve mice over a time course.

S100A9 causes mild leucocyte influx in vivo, and following intratracheal installation into murine lung and we confirmed a mild neutrophilia. In contrast to S100A8, which inhibits MC activation, S100A9 provoked activation 1–4 h post inhalation (Figure 2e) and cytokines and chemokines released from preformed stores may contribute to the leucocyte infiltration seen over 4–6 h. The rapid induction of CXCL-1 and CXCL-2 mRNA 1 h post-inhalation of S100A9 may contribute; responses to S100A8/A9 were weaker and transient. CXCL-1 and CXCL-2 are neutrophil chemoattractants and simultaneous transgenic overexpression of S100A8 and S100A9 in murine hepatocytes promoted neutrophil mobilisation via CXCL-1 induction. Our results corroborate its likely contribution seen with exogenous S100A9 and S100A8/A9 (Figure 1b). Monocytes were not recruited in response to the S100s and the CCL-2 gene was unaffected. In marked contrast to S100A8, S100A9 significantly increased endogenous CXCL-10 mRNA 1–12 h post inhalation. Only BALF from S100A9-treated mice collected 4 h post-treatment contained significantly elevated CXCL-10, and although mRNA increased over time, concentrations in BALF were low, suggesting that the amplitude of CXCL-10 induction may be regulated by a post-transcriptional mechanism, such as mRNA decay, that can influence chemokine levels. CXCL-10 recruits lymphocytes and is strongly implicated in autoimmune disorders characterised by elevated serum concentrations of S100A8/A9 and S100A9. S100A9 is a proposed therapeutic target in rheumatoid arthritis and inter-relationships between CXCL-10 and S100A9 are worthy of further investigation. Interestingly, S100A8/A9 in particular, profoundly reduced basal mRNA levels of eotaxin-2 (CCL-24; Supplementary Table 1) suggesting that it may modulate eosinophil recruitment in lung.

Protective mechanisms in innate immunity prevent excessive injury, particularly at epithelial surfaces, and the S100s have protective functions in antimicrobial and oxidative defence. Even though S100A9 and S100A8/A9 are reported TLR4 agonists, we found no fulminant expression of cytokine genes typical of an LPS-activated response. In keeping with this, inhalation of adenoviral S100A9 in TLR4-deficient mice promoted lung pathology and inflammatory cell infiltrates similar to those seen in wild-type mice, suggesting alternate pathways. Furthermore, here we show that like S100A8, S100A8/A9 and S100A9 pretreatment suppressed expression of key cytokine and chemokine genes in response to LPS challenge 2 h later. It could be argued that the S100s promote hypo-responsiveness, similar to endotoxin tolerance, but the timing of S100 pretreatment and LPS challenge renders this unlikely. Moreover, in the lung microenvironment alveolar macrophages do not become tolerant to endotoxin, repeated LPS inhalation does not reduce neutrophil recruitment and, in contrast to our findings, neutrophil chemokines CXCL-1 and CXCL-2 are not suppressed.

S100A9 inhalation substantially suppressed the high induction of chemokine genes by LPS, also reflected by CCL-2 and CXCL-10 concentrations in BALF. S100A9 also reduced TNF-α and IL-6 mRNA/protein levels but not IL-1β. IL-1β processing and secretion is dependent on activation of the NLRP3 inflammasome that can be driven by SAA. S100A9 strongly reduced basal SAA3 mRNA in naïve lung (Supplementary Table 1), and the highly elevated SAA3 gene expression induced by LPS in vivo, from 419-fold to 85-fold above baseline (Supplementary Table 2) although there was no significant change in protein concentrations in BALF; S100A8/A9 pretreatment was not suppressive. Nevertheless, both preparations suppressed neutrophil influx mediated by LPS in this study suggesting a more generalised immunosuppression that may also affect SAA-mediated responses. These results contrast markedly with S100A8, which, like dexamethasone, strongly inhibited SAA induction by LPS in ALI.

Little is known concerning IL-10 regulation in lung epithelial cells and our combined studies suggest an important new pathway. In marked contrast to S100A8, which directly stimulated high IL-10 expression in the airway epithelium and in tracheal epithelial cells in vitro, S100A8/A9 and S100A9 had no direct effect. S100A8 is a redox-sensitive protein with a single highly reactive Cys residue. Indeed, dithiol agents that restore glutathione levels in models of airway hyper-responsiveness enhance IL-10 production in the lung and attenuate disease and some consider IL-10 an anti-inflammatory anti-oxidant. Although murine S100A9 has 3 Cys residues, these are less reactive than Cys12 of S100A8 and although S100A8/A9 is not disulphide-linked, the complex did not induce IL-10, suggesting a novel structural mechanism that could modulate effects of S100A8 on redox signalling. Moreover, unlike S100A8, neither S100A8/A9 nor S100A9 induced S100A8 mRNA. IL-10 is essential for S100A8 induction in murine macrophages by TLR agonists whereas S100A9 is not induced, and like IL-10, S100A9 is expressed in alveolar macrophages of the M2-phenotype. Thus only S100A8 appears to regulate an IL-10-dependent feedback to temper inflammation.

Signalling pathways contributing to S100 suppression of ALI are likely to be complex. MKP-1 negatively regulates the MAPK pathway in LPS-initiated signalling, although levels in lungs from LPS-treated mice were significantly reduced by S100A9 and this may facilitate a more sustained activation of the MAPK pathway, and somewhat less effective immunosuppression than seen with S100A8. Although p38 and JNK are preferentially phosphorylated in response to LPS, phosphorylation levels in lungs from S100A8-treated mice did not change (not shown) and Erk phosphorylation was somewhat elevated by S100A9. The lack of prominent changes suggested alternate mechanisms.

S100A8 reduced gene induction of NF-κB and enhanced IkBα expression, and we proposed these to contribute to suppression of LPS-induced ALI. IkBα was also increased by S100A9 but at 4 h post LPS inhalation, and may modulate intermediate response genes induced by LPS. In addition, p50 subunit levels of NF-κB were significantly elevated by S100A9 pretreatment, raising the possibility...
that the p50/p50 homodimer may be involved, as described for inhibition of TNF-α production by resolvins-1-treated macrophages. SIRT1, an NAD+‐dependent protein deacetylase sirtuin-1, suppresses NF-κB signalling by deacetylating the p65 subunit. Agents such as resveratrol that elevate SIRT1 expression in mice, significantly reduce systemic levels of several cytokines (reviewed in ref. 29), and protect against endotoxin shock and sepsis. SIRT1-/- mice acquire sepsis-induced ALL, and SIRT1 is implicated in suppression of NF-κB and inflammasome activation to reduce inflammatory mediator production.

Here we show that S100A8/A9 and S100A9 restored the basal SIRT1 levels that were reduced by LPS inhalation, and propose a novel mechanism whereby regulation of SIRT1 by the S100 proteins may contribute to their anti‐inflammatory functions. Although SOCS3 moderates LPS‐induced inflammmation, we found significantly less SOCS3 mRNA induction in lungs from mice pretreated with S100A8/A9 or S100A9. Notwithstanding, p‐STAT3 was elevated in these samples. Elevated STAT3 activation contributes to epithelial protection during ALI and may be involved in the suppression by the S100 proteins. Analysis of the lung is complex and characterisation at the cellular level may provide new insights. More detailed investigation of downstream effects of the myeloid‐derived S100 proteins on these pathways, and their contributions to suppression of ALI, are warranted.

Here we demonstrate important functional differences of S100A8/A9 and S100A9 that distinguish them from S100A8. Neither influenced the S100A8/IL-10 induction pathway in the airways. On the other hand, only S100A9 directly induced CXCL-10 that may contribute to the neutrophilia seen with this protein. However all S100 preparations reduced the strong induction of chemokine and cytokine genes following LPS challenge although S100A8/A9 was least effective (ref. 11 and Supplementary Table 2). In marked contrast to cytokine genes following LPS challenge although S100A8/A9 was least effective, S100A8, S100A9 or S100A8/A9 did not inhibit MC activation in vitro, or effectively reduce SAA induction by LPS. Structural differences within these proteins may contribute to functional diversity. For example, the flexible extended C-terminal tail of S100A9 has a potential ligand‐binding domain implicated in neutrophil‐immobilising factor function. The hinge regions between the two calcium‐binding domains of S100A8 and S100A9 are structurally divergent and differences in electrostatic potential and hydrophobicity distribution around these may generate different binding affinities and/or facilitate binding to different target proteins, producing specific functions. Impacts of S100A8 and S100A9 would also depend on the type expressed, and may be context‐ and concentration‐dependent. Nonetheless, results presented here demonstrate distinct and common immunoregulatory functions for S100A8, S100A8/A9 and S100A9 in the lung. We propose that their secretion by infiltrating myeloid cells, and induction in macrophages and epithelial cells by particular agonists, may contribute to normal lung homeostasis and protection against excessive fulminant inflammation.

METHODS

Mice

Specific pathogen‐free female BALB/c mice (7–9 weeks) from Australian Bioresources Ltd (Moss Vale, Australia) were used according to ethics guidelines, with institutional approval of the Animal Care and Ethics Committee of the University of New South Wales (reference number: 13/119A).

S100 preparations

Recombinant murine S100A8 and S100A9 expressed as GST‐fusion proteins in E. coli were treated with thrombin to remove GST, then purified to homogeneity by sequential C4- and C18‐reverse‐phase high‐performance liquid chromatography, and reconstitution, validation of purity and mass of each preparation was as described. Proteins differed from the native counterparts in having an additional dipeptide, Gly‐Ser at the N‐terminus. Proteins were stored in 45% acetonitrile, 0.1% trifluoroacetic acid at ~80 °C under argon. Endotoxin levels were monitored and proteins only used if concentrations were <10 pg/10 μg S100, tested by Limulus assay (Associates of Cape Cod). To prepare the S100A8/A9 complex, equimolar amounts of S100A8 and S100A9 in HBSS were incubated for 1 h at 4 °C under argon.

Endotoxin minimisation

Where possible, all work was performed in a pre‐sterilised fume hood. Incubations were in dedicated LPS‐minimised incubators. All tissue culture plates, tubes and so on were only used if stated to contain <2.0 endotoxin units/item, otherwise were gamma‐irradiated. Glassware for preparation of media or buffers was treated with 0.1% NaOH, rinsed with sterile water autoclaved, and baked at 250 °C for 30 min to vapourise remaining endotoxin. Buffers that were not endotoxin free were Zetapore filtered. For high‐performance liquid chromatography preparation of recombinant S100s, columns were pre‐flushed with endotoxin‐minimised buffers and fractions collected into Nunc minisorb tubes (Nunc Roskilde Denmark) which do not absorb these very hydrophobic proteins and when tested by us, do not contain traces of endotoxin.

Assessment of effects on inflammation

To define direct effects of the S100s (10 μg/50 μl HBSS unless otherwise stated), these were administered onto the nares and effects in lungs from mice sacrificed 1, 4, 6, 12 or 20 h post‐inhalation as described. Inflammation induced by LPS (E. coli, serotype 055:B5, Sigma‐Aldrich, St. Louis, MO, USA) was with 10 μg/50 μl PBS administered intranasally. Intranasal S100s (10 μg/50 μl HBSS) were given 2 h before LPS; control mice received equal volumes of vehicle (HBSS+PBS). Mice were killed 4 h post LPS inhalation, or at 1 h where indicated. Portions of right lung preserved in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) were stored at 4 °C until RNA was extracted; the remainder stored at ~80 °C to be used for protein extraction and analysis. Left lungs were fixed in 10% neutral‐buffered formalin.

Analysis of infiltrating cells and mast cell degranulation

Harvest of BALF and enumeration of leukocytes therein were as described. β‐hex activity in BALF was used as a measure of MC degranulation as described. Cell numbers, and MC that were degranulating, were manually counted by a blinded operator.

RT-qPCR array

Total RNA preparation and PCR amplification was as described. Based on our previous report on effects of S100A8 on ALI, 46 genes mediating acute inflammation, and 3 housekeeping genes, were chosen for this comparative study (Supplementary Tables 1 and 2). Data analysis was performed essentially as described using the web‐based software package and Excel‐based analysis tool (SA Biosciences, Qiagen, Chadstone Centre, VIC, Australia). The HPRT, β‐actin and GAPDH were set as housekeeping genes (geometric mean) and all Cq (cross point) values were set to a cut‐off threshold of 37. Baseline levels of genes in mice treated with vehicle (HBSS and PBS), and results for LPS alone, and S100A9 or S100A8 were reported. Data files were lodged in the Gene Expression Omnibus (accession no. GSE74129; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token = kvkhuououyhnkn&acc = GSE74129).

Immunohistochemistry

Serial sections of formalin‐fixed, paraffin‐embedded lung tissue (4 μm) were stained with hematoxylin and eosin or anti‐S100A8 or anti‐S100A9 and imaged as described. Rabbit anti‐S100A8 IgG does not cross‐react with S100A9; anti‐S100A9 IgG reacts with S100A9 and S100A8/A9 complex.
Enzyme-linked immunosorbent assay
IL-1β, IL-4, IL-6, IL-10, TNF-α, CCL-2 and CXCL-10 enzyme-linked immunosorbent assay kits were from R&D Systems (Minneapolis, MN, USA), and SAA3 from MyBioSource (San Diego, CA, USA) and used according to manufacturer’s instructions.

Western blotting
Lysates of lung homogenates (20 µg protein) were subjected to Western blotting as described.11 The following antibodies were used: rabbit monodonal anti-phospho-MEK1/2 (Ser217/221, 1:1000 v/v), anti-phospho-p38 MAPK (Thr180/Tyr182, 1:1000 v/v), anti-IκBα (1:1000 v/v) and NF-κB1 p105/p50 (1:1000 v/v), and rabbit polyclonal anti-MEK1/2 (1:1000 v/v), anti-phospho-Erk1/2 MAPK (Thr202/Tyr204, 1:1000 v/v), anti-SIRT1 (1:1000 v/v), anti-β-actin (1:500 v/v, rabbit polyclonal, Thermo Fisher Scientific) was used to control for protein loading.

Mediator induction by S100A8 and S100A9 in tracheal epithelial cells in vitro
Primary epithelial cells from excised trachea were established using a modified method of Hiroshima et al.47 Tracheas cut into 0.5–1 mm fragments and immobilised as explants on collagen I-coated wells (5 µg cm−2, collagen 1 in 0.1 M acetic acid) were cultured for 2 days at 37 °C in 5% CO2 in air in Dulbecco’s modified eagle medium supplemented with 10% foetal bovine serum, 100 units per ml penicillin, 100 µg ml−1 streptomycin and 0.25 µg ml−1 amphotericin B (all from Thermo), then for 20 days in keratinocyte-serum-free medium (SM) supplemented with 30 µg ml−1 bovine pituitary extract, 0.2 ng ml−1 epidermal growth factor and 100 µg ml−1 kanamycin (all from Thermo). After outgrowth, cells were cultured until the second passage.

Statistical analysis
Lymphocyte and neutrophil numbers in time course experiments were analysed using the Student’s t-test. Comparisons of mRNA levels between groups and statistical significance was set at 2.5-fold) and -log10 of P-values were calculated using a Student’s t-test of the 2−ΔΔct method, significance set at 2−5−fold and -log10 of P-values comparing S100A9 with S100A8/A9-treated mice (Student’s t-test, set at P<0.05) for each gene. Clustergrams and volcano plots were generated by web-based software (SA Biosciences).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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