IL-1 receptor regulates S100A8/A9-dependent keratinocyte resistance to bacterial invasion

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Previously, we reported that epithelial cells respond to exogenous interleukin (IL)-1α by increasing expression of several genes involved in the host response to microbes, including the antimicrobial protein complex calprotectin (S100A8/A9). Given that S100A8/A9 protects epithelial cells against invading bacteria, we studied whether IL-1α augments S100A8/A9-dependent resistance to bacterial invasion of oral keratinocytes. When inoculated with *Listeria monocytogenes*, human buccal epithelial (TR146) cells expressed and released IL-1α. Subsequently, IL-1α-containing media from *Listeria*-infected cells increased *S100A8/A9* gene expression in naïve TR146 cells an IL-1 receptor (IL-1R)-dependent manner. Incubation with exogenous IL-1α decreased *Listeria* invasion into TR146 cells, whereas invasion increased with IL-1R antagonist. Conversely, when *S100A8/A9* genes were knocked down using short hairpin RNA (shRNA), TR146 cells responded to exogenous IL-1α with increased intracellular bacteria. These data strongly suggest that infected epithelial cells release IL-1α to signal neighboring keratinocytes in a paracrine manner, promoting S100A8/A9-dependent resistance to invasive *L. monocytogenes*.

INTRODUCTION

Lining the oral meatus of the digestive tract, keratinocytes are the first mucosal epithelial cells to provide barrier protection against both oral and ingested enteric pathogens. Forming a stratified tissue, oral keratinocytes contribute to innate protection of underlying tissues through continuous exfoliation and production of antimicrobial proteins (AMPs). AMPs released onto the mucosal surface and into the salivary milieu contribute to the control of oral pathogens and commensals. AMPs within keratinocytes also appear to increase resistance to bacterial invasion.[1–3]

S100A8/A9 is constitutively expressed within oral epithelial cells[4,5] and upregulated during gingival inflammation,[6] whereas S100A8/A9 is not expressed in uninflamed intestinal epithelium,[7] but is induced during inflammatory bowel disease.[8,9] Potentially protective during gastrointestinal infection and inflammation, S100A8/A9 expression increases in response to *Salmonella* in several animal models.[10–13] A heterodimeric complex of S100A8 and S100A9 (MRP8 and MRP14), calprotectin appears unique among the AMPs since it can be released[14] and active extracellularly[15–17] and also function within the cytoplasm of keratinocytes.[3] As an intracellular AMP, we have shown that S100A8/A9 increases epithelial cell resistance to invasion by both oral and enteric bacterial pathogens, including *Porphyromonas gingivalis*, *Salmonella typhimurium*, and *Listeria monocytogenes*.[3,18,19]

In response to invasive pathogens, epithelial cells also release proinflammatory cytokines and chemokines, which appear to alert and recruit innate immune cells to the site of infection. For example, patients with periodontal disease show increased gingival tissue and crevicular fluid levels of interleukin (IL)-1α and IL-1β, IL-6, IL-10, and CXCL8.[20,21] Since oral epithelial cells do not express functional IL-1β,[22] oral keratinocytes more likely rely on producing IL-1α as a functional mediator of innate immunity.[23] The contribution of active IL-1β in periodontal disease, therefore, is mostly likely from cells of non-epithelial origin. To better understand early innate immune responses of the oral epithelium, we investigated whether keratinocytes could autonomously use IL-1α in modulating the epithelial barrier against invasive microorganisms.

Released IL-1α could signal the presence of pathogens to oral epithelial cells via the surface IL-1 receptor I (IL-1RI). As we reported,[24] IL-1α signals keratinocytes to express several AMPs, including defensins, LL-37, secretory leukocyte protease inhibitor, and S100A8/A9. Similarly, pathogens directly increase expression and release of AMPs by keratinocytes.[25–27] Yet, it is unknown whether oral keratinocytes autonomously alert neighboring keratinocytes to the presence of pathogenic

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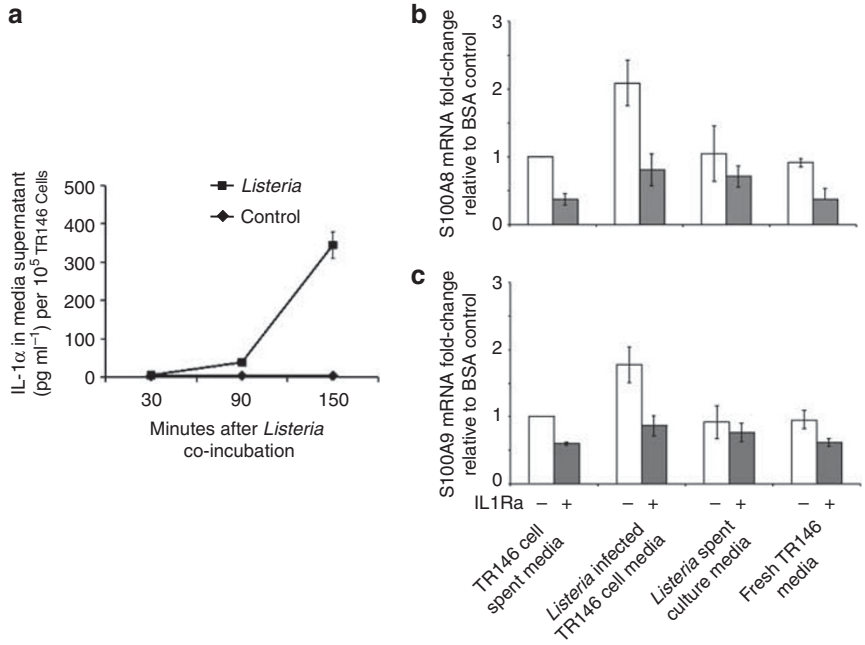

Figure 1 TR146 buccal epithelial cells (carcinoma) express *S100A8/A9* in response to released interleukin (IL)-1α during *L. monocytogenes* infection. (**a**) TR146 cells release IL-1α in a time-dependent manner in response to *L. monocytogenes* (■) and compared with no bacteria control (◆) using ELISA. IL-1α release data shown are the mean of three replicates of one representative experiment ±s.d. (**b**) *S100A8* and (**c**) *S100A9* mRNA expression in response to fresh, sterile-filtered TR146 media, spent TR146 media from oral keratinocytes, spent culture media recovered after growth of *Listeria*, or spent TR146 media recovered after co-incubation with *L. monocytogenes* for 2.5 h. As indicated, all cells were incubated in bovine serum albumin (BSA)-containing media with or without IL-1Ra. IL-1Ra blocks cell responses to endogenously produced and released IL-1α. For panels **b** and **c**, data shown are the mean fold change in gene expression relative to BSA controls ± s.e.m. of two independent experiments as determined by qRT-PCR.

bacteria and promote innate intracellular resistance to invasion. We hypothesized that oral mucosal epithelial cells use IL-1α to increase S100A8/A9-dependent resistance to invasive bacterial pathogens. Using a human buccal epithelial cell line (TR146), we characterized the mechanism of resistance to invasion of *L. monocytogenes*. In response to *L. monocytogenes*, previously naïve TR146 cells release IL-1α to increase *S100A8/A9* gene and protein expression in an IL-1RI-dependent manner. The IL-1α-dependent increase in expression of S100A8/A9 promoted keratinocyte resistance to invading *Listeria*.

RESULTS

IL-1α signaling by oral keratinocytes in response to *L. monocytogenes*

In response to *L. monocytogenes* 10403S (multiplicity of infection (MOI) of 100), TR146 buccal epithelial cells release 344 pg ml^{-1} IL-1α per 10^5 cells within 150 min (**Figure 1a**). When incubated with naïve TR146 cells, media from *Listeria*-infected TR146 cells increased expression of *S100A8* (**Figure 1b**) and *S100A9*-specific mRNAs (**Figure 1c**) in an IL-1 receptor antagonist (IL-1Ra)-inhibitable manner at 24 h. In contrast, spent media from *Listeria* or TR146 cells alone failed to show IL-1Ra-inhibitable increases in *S100A8* and *S100A9* expression.

IL-1α regulation of *S100A8/A9*

TR146 and HaCaT cells were incubated with increasing amounts of IL-1α and the expression of *S100A8*- and *S100A9*-specific mRNAs was determined at 24 h. TR146 cells maximally express *S100A8/A9* subunits in the presence of 1 ng ml^{-1} exogenous IL-1α (**Figure 4b**), while heat denatured IL-1α failed to induce *S100A8/A9* expression (*data not shown*). In response to 1 ng ml^{-1} exogenous IL-1α, TR146 cells increased *S100A8/A9* expression in a time-dependent manner over 48 h (*data not shown*). In contrast, as we reported, human epidermal cells (HaCaT)[24] maximally upregulate *S100A8* and *S100A9* in response to 10 ng ml^{-1} IL-1α.

IL-1α regulation of S100A8/A9 is IL-1 receptor dependent

Using recombinant IL-1Ra, we determined whether exogenous IL-1α signaled through surface IL-1RI to induce *S100A8/A9* gene expression. TR146 and HaCaT cells were incubated with IL-1α (1 and 10 ng ml^{-1}, respectively) and 10 or 100 ng ml^{-1} recombinant IL-1Ra. IL-1Ra inhibited IL-1α-induced *S100A8* and *S100A9* gene expression at 24 h in a dose-dependent manner in both TR146 (**Figure 2a**) and HaCaT cells (**Figure 2b**). The HaCaT and TR146 cell responses to exogenous IL-1α may reflect differing *IL1-RI* expression in the two lines. TR146 cells showed 4-fold more *IL1-RI*-specific mRNA than HaCaT cells, but only a 1.1-fold increase in IL-1RI protein (**Figure 2c,d**).

IL-1α-dependent upregulation of S100A8/A9 antagonizes invasion by *L. monocytogenes*

Next, we determined whether exogenous IL-1α acting through the IL-1 receptor on TR146 cells induces sufficient S100A8/A9 to antagonize invasion by *L. monocytogenes*. Using optimized conditions for S100A8/A9 expression, TR146 cells were incubated

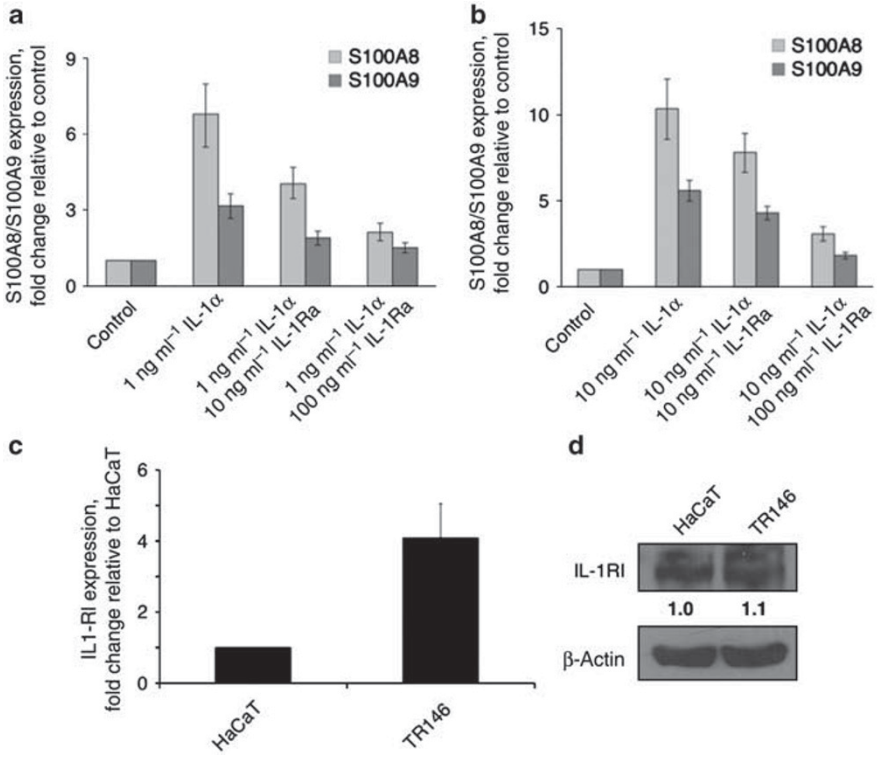

Figure 2 *S100A8/A9* gene expression in epithelial cells is interleukin (IL)-1 receptor dependent. (**a**) IL-1α-induced expression of *S100A8* and *S100A9* genes in TR146 cells in the presence or absence of IL-1 receptor antagonist (IL-1Ra) as determined using qRT-PCR at 24 h. (**b**) As in panel **a**, except HaCaT cells were used. qRT-PCR data shown are the mean fold change relative to bovine serum albumin (BSA) controls ± s.e.m. of two independent experiments. (**c**) Expression of *IL-1 receptor I* (*IL-1RI*) in HaCaT and TR146 cells as determined using qRT-PCR at 24 h. (**d**) IL-1RI immunoreactive proteins from HaCaT and TR146 whole cell lysates as visualized by immunoblotting. As quantified using ImageJ, IL-1RI protein expression is shown below each immunoblot as fold increase relative to HaCaT after normalizing to β-actin. All experiments were repeated three times.

for 24 h with 1 ng ml^{-1} IL-1α with or without IL-1Ra. As expected, expression of *S100A8* and *S100A9* genes (**Figure 3a**) and proteins (1.8- and 1.9-fold, respectively; **Figure 3b**) increased in an IL-1RI-dependent manner. In identical conditions, exogenous IL-1α was associated with up to a 39% (**$P < 0.01$) reduction of *L. monocytogenes* invasion into TR146 cells as compared with bovine serum albumin (BSA) control (**Figure 3c**). In the absence of exogenous IL-1α, IL-1Ra also prevented increased S100A8/A9 expression and supported significantly more *Listeria* invasion than BSA control (27% increase in recovered intracellular colony forming units (CFU); **$P < 0.01$), suggesting that released IL-1α increases resistance to invasion.

IL-1α-mediated resistance to *L. monocytogenes* invasion is S100A8/A9 dependent

Using short hairpin RNAs (shRNAs) against both *S100A8* and *S100A9*, we constructed stable knockdowns in TR146 cells (A8A9c10). Relative to cells that express non-specific shRNAs (Neg3), the A8A9c10 line suppressed S100A8 and S100A9 to ~10% of wild-type and 15% of Neg3 levels (**Figure 4a**). To determine whether wild-type TR146 and Neg3 cells respond similarly to exogenous IL-1α, we compared the expression of *S100A8* and *S100A9* using qRT-PCR. In response to exogenous IL-1α, wild-type TR146 (**Figure 4b**) and Neg3 (**Figure 4c**) cells upregulate *S100A8/A9* genes in a dose-dependent manner. Suggesting similar transcriptional regulation, exogenous IL-1α also induced expression of *β-defensin 2* (*h-BD2*) in wild-type (**Figure 5a**), Neg3 and A8A9c10 cells (**Figure 5b**); in all lines, IL-1α-induced upregulation of *h-BD2* was inhibited by IL-1Ra (**Figure 5c**,**d**).

Exogenous IL-1α tended to reduce resistance against *L. monocytogenes* invasion (more intracellular CFUs recovered) in A8A9c10 cells when compared with Neg3 cells ($P = 0.06$; **Figure 6a**), which was confirmed in a second *S100A8/A9* knockdown clone (A8A9c27) (*$P < 0.05$; **Figure 6b**). Consistent with specific silencing of *S100A8/A9*, A8A9c10 and A8A9c27 cell lines showed similar decreased resistance to *L. monocytogenes* invasion in the presence and absence of IL-1α. Unlike wild-type TR146 cells, Neg3 maximally expressed S100A8/A9 in the presence of 10 ng ml^{-1} IL-1α (**Figure 4b,c**), which tended to reduce invasion (increase resistance) by 23% ($P = 0.09$), whereas A8A9c10 showed a 23% increase (*$P < 0.05$) in intracellular *L. monocytogenes* as compared with BSA controls (**Figure 6c**). When compared with Neg3 cells, IL-1α-treated A8A9c10 cells showed 60% greater intracellular CFU (*$P < 0.05$; **Figure 6c**). Resistance to *L. monocytogenes* invasion was directly associated with S100A8 and S100A9 protein expression. In Neg3 cells, S100A8 and S100A9 proteins were increased 2.3- and 1.6-fold, respectively, in response to exogenous IL-1α, whereas in A8A9c10 S100A8/A9 knockdown cells, S100A8 and S100A9 protein increased 1.7- and 2.7-fold, respectively (**Figure 6d**).

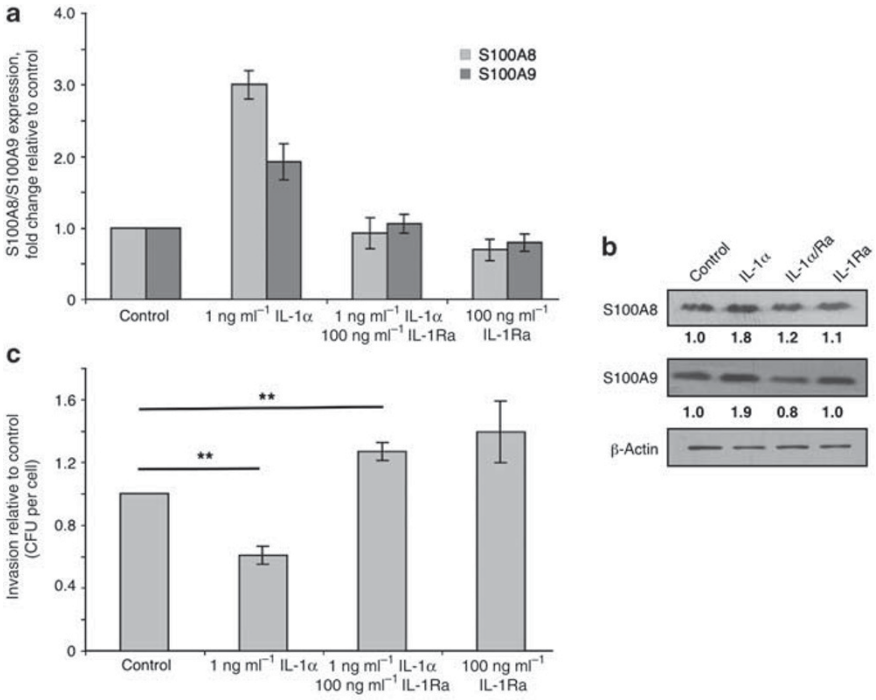

Figure 3 Exogenous interleukin (IL)-1α enhances keratinocyte resistance to *L. monocytogenes* invasion. (**a**) *S100A8* and *S100A9* gene expression in TR146 cells in response to IL-1α in the presence and absence of IL-1 receptor antagonist (IL-1Ra) at 24 h as compared with bovine serum albumin (BSA) control. (**b**) S100A8 and S100A9 immunoreactive proteins from TR146 whole cell lysates in response to IL-1α with and without IL-1Ra as visualized by immunoblotting. As quantified using ImageJ, S100A8 and S100A9 protein expression is shown below each immunoblot as fold increase relative to BSA control after normalizing to β-actin. (**c**) *L. monocytogenes* invasion into TR146 cells as determined by antibiotic protection assay (intracellular recovered colony forming units (CFU)). Data shown are the mean intracellular CFUs per cells ± s.e.m.; $^{**}P<0.01$. All experiments were conducted in triplicate and repeated three times.

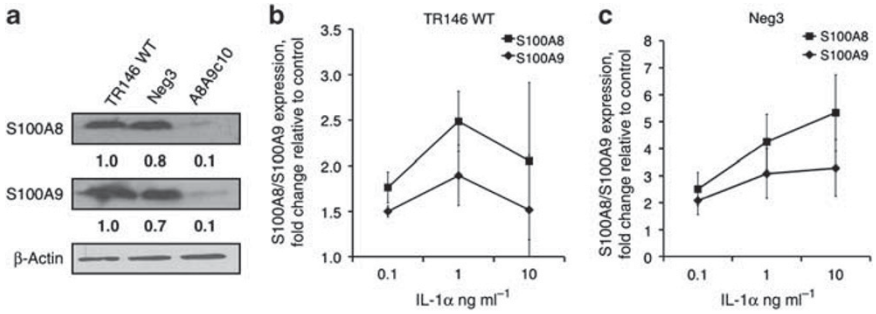

Figure 4 Characterization of S100A8/A9 knockdown in TR146 cells. (**a**) Immunoreactive S100A8 and S100A9 in wild-type TR146, Neg3 cells (expressing shRNA not specific to any known human gene) and A8A9c10 (*S100A8* and *S100A9* knocked down). Densitometric quantification of S100A8 and S100A9 protein expression was normalized to β-actin using ImageJ and displayed below the immunoblot as fold increase relative to wild-type TR146 cells. (**b**) *S100A8* (■) and *S100A9* (◆) gene expression in response to exogenous IL-1α after 24 h in wild-type TR146 cells. (**c**) *S100A8* (■) and *S100A9* (◆) gene expression in response to exogenous IL-1α at 24 h in Neg3 cells. All experiments were conducted in triplicate and repeated at least two times. Data shown are the mean fold change relative to means ± s.e.m.

After 2 h of incubation, *Listeria* CFU recovery from culture media of Neg3 and A8A9c10 cells was similar and unaffected by IL-1α incubation; invasion was independent of keratinocyte cell size (*data not shown*).

DISCUSSION

In this report, we dissect a mechanism by which mucosal epithelial cells use IL-1α as a signaling molecule to respond and protect themselves against infection by the pathogenic bacterium, *L. monocytogenes*. We also show that the protection against invasion by *L. monocytogenes* is cell autonomous, whereby epithelial cells confer resistance to the pathogen independently of dedicated innate and adaptive immune cells. In response to *L. monocytogenes*, epithelial cells release IL-1α and, in an IL-1RI-dependent manner, modulate autonomous resistance to these invasive enteric pathogens. Other invasive mucosal pathogens including *C. albicans*[23] and *Porphyromas gingivalis*[25,28,29] also induce the release of IL-1α from oral keratinocytes over time, suggesting that the epithelial response to pathogens could be generalized.

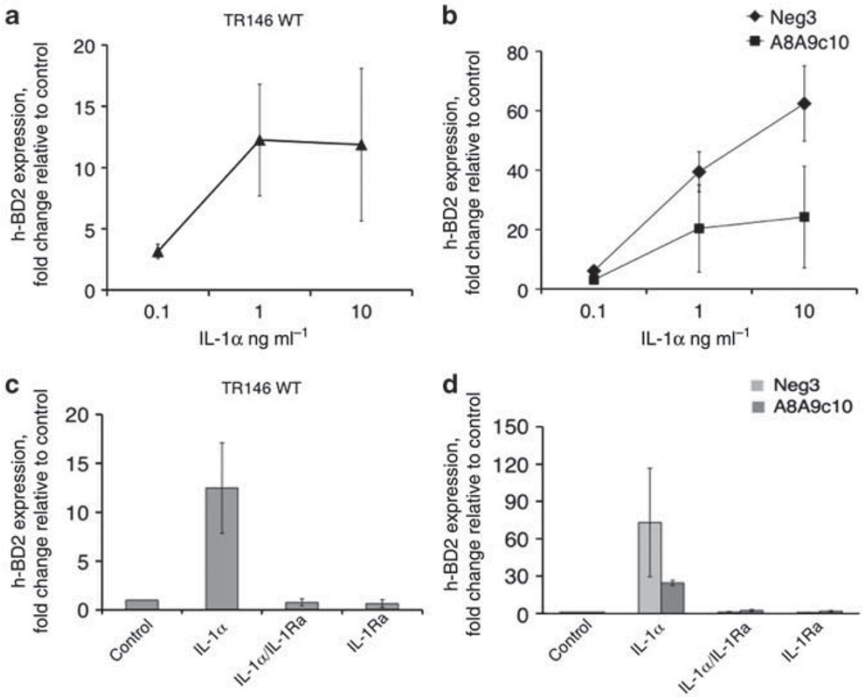

Figure 5 Expression of *h-BD2* by wild-type and transfected TR146 cells in response to interleukin (IL)-1α. (**a**) *h-BD2* gene expression in wild-type TR146 cells at 24 h in response to increasing exogenous IL-1α as determined using qRT-PCR. (**b**) As in panel **a**, except that Neg3 (◆) and A8A9c10 (■) cells are studied. (**c**) *h-BD2* expression at 24 h in wild-type TR146 cells in response to IL-1α in the presence and absence of IL-1Ra. (**d**) As in panel **c**, except that Neg3 and A8A9c10 cells are studied. All experiments were conducted in triplicate and repeated two times. Data shown are the mean fold change relative to bovine serum albumin (BSA) means ± s.e.m.

Upon invasion into keratinocytes, *L. monocytogenes* and *P. gingivalis* induce calcium release from intracellular stores,[30,31] which can activate the calcium-dependant protease, calpain. Activated calpain cleaves the 32-kDa proIL-1α to produce mature 17 kDa IL-1α, a critical step during Golgi-independent release of IL-1α. Release of the IL-1α propeptide also occurs apparently through non-canonical plasma membrane translocation.[32,33] Both the propeptide and mature IL-1α are recognized by the IL-1RI,[34] which is generally expressed on the surface of mucosal epithelial cells.[35] Spent media from *Listeria*-infected oral keratinocytes increased *S100A8/A9* expression in an IL-1RI-dependent manner, suggesting that released IL-1α was biologically active. Hence, IL-1α can alert or signal neighboring epithelial cells to nearby stresses in a paracrine manner.

We had hypothesized that mucosal epithelial cells use IL-1α as a signaling molecule to increase S100A8/A9-dependent resistance to *Listeria* invasion in oral keratinocytes. We examined whether exogenous IL-1α induces expression of *S100A8/A9* and other AMP genes in an IL-1RI-dependent manner. In TR146 cells, exogenous IL-1α induces dose-dependent upregulation of representative AMPs, including both *S100A8/A9* subunits and *h-BD2*, consistent with our previous studies in HaCaT epithelial cells.[24,36] The increased concentration of IL-1α required to maximally express *S100A8/A9* in HaCaT as compared with TR146 cells appears to be a result of greater IL-1RI expression, suggesting that keratinocytes could act as sensitive sentinels in the oral mucosa. Expression of *S100A8/A9* and *h-BD2* by TR146 cells was maximal in response to 1 ng ml^{-1} IL-1α, which appears to be biologically relevant since *L. monocytogenes* induced TR146 cells to release ~1.4 ng ml^{-1} IL-1α into the media. Hence, IL-1α released from epithelial cells (endogenous) or added to the cells (exogenous) can signal naïve keratinocytes through IL-1RI to induce expression of S100A8/A9 and other AMPs. Although also biologically active within the cell,[37,38] intracellular IL-1α is insensitive to IL-1Ra,[39] which effectively blocks extracellular IL-1α from the IL-1 type I receptor.[40–42] Since either spent culture media containing IL-1α or added IL-1α appears to signal epithelial cells similarly, we concluded that exogenous IL-1Ra targets surface IL-1RI; increased AMP gene expression is dependent on surface IL-1α/IL-RI interactions.

IL-1R-dependent responses have been implicated in resistance to bacterial[41,43,44] and fungal infections[45,46] *in vitro* and in animal models. To determine whether IL-1RI contributes to resistance of TR146 cells to infection by *L. monocytogenes*, cells were incubated for 24 h with IL-1α in the presence or absence of IL-Ra. After infection with *Listeria*, an antibiotic protection assay was performed to discriminate extracellular and invaded bacteria. Exogenous IL-1α was shown to increase production of S100A8/A9 about 2-fold (**Figure 3a**,**b**), which, for the first time, was shown to be sufficient to augment keratinocyte resistance to invasive *Listeria* (**Figure 3c**). Moreover, since S100A8 and S100A9 do not form homodimers within cells, the increase in S100A8/A9 protein expression is expected to reflect functional S100A8/A9 complex.[47] In the presence or absence of exogenous IL-1α, IL-1Ra reduced S100A8/A9 expression, increasing

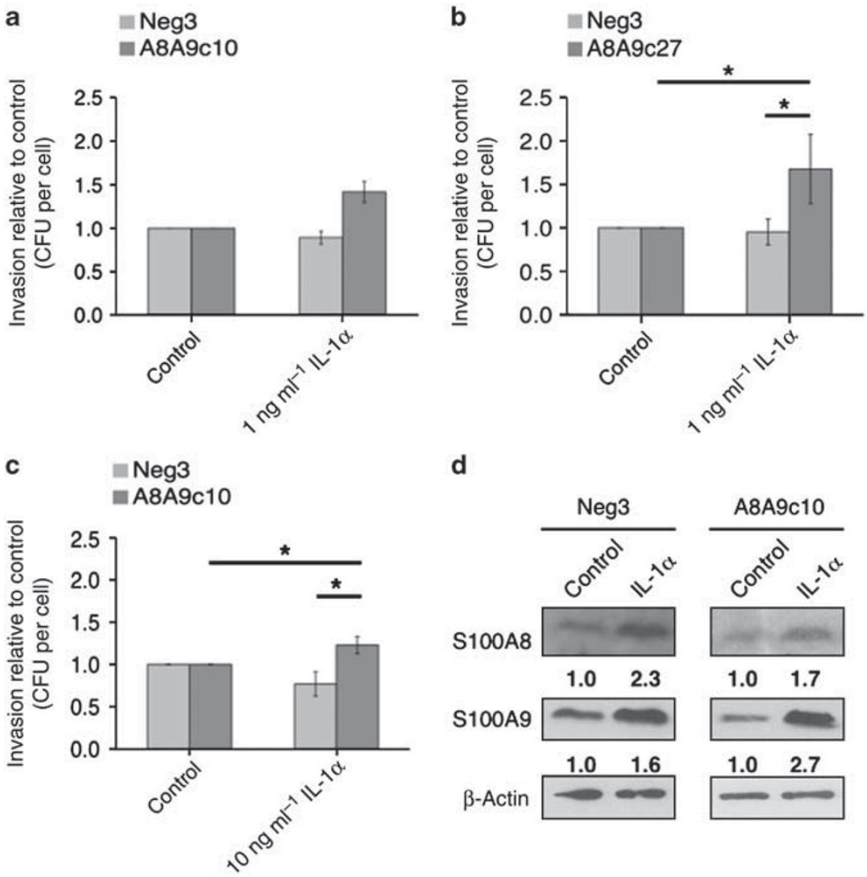

Figure 6 Interleukin (IL)-1α-mediated resistance to *Listeria* invasion is S100A8/A9 dependent. Cells were incubated in the presence or absence of IL-1α for 24 h and then infected with *Listeria* for 2 h. (**a**) *Listeria* invasion into A8A9c10 and Neg3 cells in presence of 1 ng ml^{-1} IL-1α. Data shown are the mean invasion relative to bovine serum albumin (BSA) control for six experiments, each in triplicates ± s.e.m. (P=0.06). Intracellular recovered colony forming units (CFU) were determined by antibiotic protection assay. (**b**) *Listeria* invasion into clone A8A9c27 (similar levels of S100A8 and S100A9 expression to A8A9c10) and Neg3 cells in response to 1 ng ml^{-1} IL-1α (means ± s.e.m., N=2; *P<0.05). (**c**) *Listeria* invasion into A8A9c10 and Neg3 cells after 24 h in response to 10 ng ml^{-1} IL-1α (means ± s.e.m., N=3; *P<0.05). (**d**) Immunoreactive S100A8 and S100A9 subunit proteins expressed in response to BSA control or 10 ng ml^{-1} IL-1α at 24 h in Neg3 and A8A9c10 cells as detected by Western blotting. As quantified using ImageJ, S100A8 and S100A9 protein expression is shown below each immunoblot as fold increase relative to BSA control after normalizing to β-actin.

the number of recoverable invaded *Listeria* and reducing resistance to invasion. Since endogenous IL-1α is released by infected oral keratinocytes (**Figure 1a**), blocking IL-1R1 with IL-1Ra appeared to reduce protection of epithelial cells mediated by S100A8/A9.

To show more definitively that resistance to invasion was mediated by S100A8/A9, we knocked down S100A8 and S100A9 subunit proteins using shRNA. S100A8/A9 was reduced nearly 85% in clone A8A9c10 when compared with the sham silenced Neg3 cells (**Figure 4a**). Whereas expression of *h-BD2* in response to IL-1α was not affected, *h-BD2* expression in response to increasing doses of IL-1α was similar in TR146 cells (**Figure 5a**) and A8A9c10 (**Figure 5b**). In response to IL-1α, expression of *h-BD2* by Neg3 cells was anomalously high and Neg3 resistance to invasion in some experiments appeared to be unaffected by IL-1RI stimulation. The inability of IL-1α to induce resistance in Neg3 cells may be the result of shRNA transfection, which can induce non-specific responses in mammalian cells as previously reported.[48,49] While S100A8/A9-mediated resistance to invasion by *Listeria* appeared lower in A8A9c10 cells than in Neg3 cells (**Figure 6a**), a second silenced clone, A8A9c27 showed significantly less resistance to invasion than Neg3 cells (**Figure 6b**). The increased h-BD2 in Neg3 cells could be argued to have increased the resistance of these cells to invasion in the presence of IL-1α, but this does not appear to be the case. Neg3 cells (**Figure 6b,c**) and wild-type TR146 cells (**Figure 3c**) showed similar reductions in invaded *Listeria* in the presence of IL-1α. We conclude therefore that IL-1α increases expression of S100A8/A9, which specifically protects the epithelial cell against invasion by *Listeria*, supporting our hypothesis that IL-1α-mediated resistance to *Listeria* invasion is S100A8/A9 dependent.

We are currently pursuing the question of how S100A8/A9 controls intracellular resistance to invasion. Within the cell, S100A8/A9 has other activities that could contribute mechanistically to resistance to invasion. In HaCaT cells, overexpression of S100A8/A9 appeared to increase NADPH activity when incubated with phorbol 12-myristate 13-acetate,[50] perhaps enhancing formation of reactive oxygen species and microbial resistance to infection.[51,52] The S100A9 C-terminal extended

peptide domain may have a regulatory role in resistance to *Listeria* infection since truncated S100A9 enhances keratinocyte resistance to *Listeria* invasion.[3] This finding does not appear to support an NADPH/S100A8/A9-dependent mechanism of antimicrobial resistance since a similar mutation in S100A9 by Benedyk *et al.*[50] showed decreased NADPH activity when compared with full-length S100A9. Other structural motifs within S100A9 also appear to have a role in resistance to invasion. We have reported that S100A8/A9 requires intact EF hands to coordinate calcium binding, which could contribute to control of invasion by *Listeria* in oral epithelial cells.[3] In addition to direct antimicrobial activity, S100A8/A9 is a putative inhibitor of casein kinases I and II,[53] which mediate IL-1RI signaling in intestinal epithelial cells.[54] By regulating activity of casein kinases I and II, S100A8/A9 could modulate IL-1RI signal transduction and downstream effectors of resistance to invasion.

Over 700 species of microorganisms colonize the oral cavity[55] and nearly all respiratory and enteric pathogens also must pass through the oral cavity and do not cause infection. Although transient enteric pathogens such as *L. monocytogenes* and *Salmonella enterica serovar typhimurium* invade oral epithelial cells *in vitro*,[2] these bacteria do not cause persistent oral infections in people. Unlike unilayered cuboidal epithelial cells of the intestinal mucosa, oral keratinocytes form a stratified epithelium, which as we show can respond and resist infection by bacterial pathogens autonomously, independent of infiltrating immune cells. Since myeloid cells can also release IL-1α during inflammation, exogenous IL-1α and the autonomous responses of the epithelium may generally increase production of AMPs, including S100A8/A9, to augment the innate protective barrier to infection. We speculate that this mechanism antagonizes colonization and infection by enteric intracellular pathogens in the human oral cavity.

Periodontal disease is an irreversible loss of the connective and hard tissues underlying the oral mucosa. Initiated by dental plaque colonizing the epithelial interface approximating the tooth, pathology generally reflects propagation of infiltrating inflammatory cells in response to invasive microorganisms. Neutrophils and macrophages induce resorption of the alveolar bone and loss of connective tissue attachment to the tooth to create the characteristic space or pocket between the proximal infected epithelium and the tooth, and loss of alveolar bone. Understanding how oral keratinocytes regulate S100A8/A9 and other AMPs to increase resistance to microbial infection would appear to advance our understanding of how the host resists loss of periodontal tissues and other mucosal infections.

METHODS

Cell lines. Two S100A8/A9-expressing human keratinocyte lines were used in this study: immortalized epidermal cells, HaCaT, and buccal carcinoma cells, TR146. TR146 buccal epithelial cells have been used previously to model the oral epithelium *in vitro*.[56,57] HaCaT cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and TR146 cells in DMEM/HAMS F-12 (CellGro, Manassas, VA), supplemented with 10% fetal bovine serum and maintained at 5% CO_2 at 37 °C. TR146 cells were provided by Dr Reuben Lotan, University of Texas, MD Anderson Cancer Center, Houston, TX. HaCaT cells were provided by Dr Carol Lange, University of Minnesota, Minneapolis, MN.

Derivation of stable knockdown of S100A8/A9 in oral carcinoma cell line. TR146 cells were transfected using the GeneEraser short hairpin RNA Mammalian Expression Vectors (Stratagene, Cedar Creek, TX) to produce shRNA against the coding regions of the S100A8/A9 subunit genes *S100A8* and *S100A9*. Oligos for the sense and antisense strands of each gene were selected according to manufacturers' guidelines and cloned into the pGE-1 shRNA plasmid, containing neomycin resistance. The sense oligo sequences for S100A8 were 5′-GATCCCGAGTTGGATATCAACACTGATGGTGC AGTGAAGCTTGACTGCACCATCAGTGTTGATATCCA ACTCTTTTTT-3′ and antisense 5′-GATCCCGCAGCTGAGCTTC GAGGAGTTCATCATGCGAAGCTTGGCATGATGA ACTCCTCGAAGCTCAGCTGCTTTTTT-3′. For S100A9, the sense oligo was 5′-CTAGAAAAAAGAGTTGGATATCAACACTGATGGT GCAGTCAAGCTTCACTGCACCATCAGTGTTGATATCC AACTCGG-3′ and the antisense was 5′-CTAGAAAAAAGCAGCTG AGCTTCGAGGAGTTCATCATGCCAAGCTTCGCATG ATGAACTCCTCGAAGCTCAGCTGCGG-3′. To produce a negative control for *S100A8/A9* gene suppression, a pGE-1-negative control vector was used to express shRNA not specific for any mammalian gene. Four sham knockdown clones were screened for basal expression of S100A8 and S100A9 and compared by qRT-PCR with wild-type TR146 cells. One clone, Neg3 was selected as a negative knockdown control as *S100A8/A9* gene expression was most similar to wild-type TR146 cells (*data not shown*). We then screened six S100A8/A9 double-knockdown clones by qRT-PCR and selected two, A8A9c10 and A8A9c27, based on suppression of S100A8 and S100A9 relative to Neg3 cells. Clones that grew in the presence of 250 μg ml^{-1} G418 were selected. *S100A8/A9* gene expression was quantified by qRT-PCR and protein production was estimated by western blot. Neg3 and A8A9c10 were maintained in 250 μg ml^{-1} G418 sulfate (Geneticin, Mediatech, Herndon, VA), whereas non-transfected cells were maintained without G418 sulfate. Before use in assays, cell lines were pre-conditioned and cultured in complete medium without G418 sulfate for 2 days.

Bacteria. *L. monocytogenes* ATCC 10403S (provided by Dr Daniel Portnoy, University of California, Berkley, CA) was used as a model invasive bacterial pathogen due to its well-studied mechanism of invasion in host tissues.[58] *L. monocytogenes* was grown in brain heart infusion medium (Difco, Detroit, MI) with 1% yeast extract as previously described.[3] Briefly, overnight *L. monocytogenes* cultures were diluted 1:5 in fresh broth and subcultured for 2 more hours. Log phase *L. monocytogenes* was collected and diluted to an optical density of 0.2 at 620 nm, which corresponds to ~5.3×10^8 CFU ml^{-1}. Bacteria were washed and diluted to an MOI of 100 relative to the number of TR146 cells in each experimental group (see below, Antibiotic Protection Assay).

IL-1α release. *L. monocytogenes* was harvested at log phase, washed, and incubated for 2.5 h at MOI 100 with 4×10^5 wild-type TR146 cells in culture media as described in *Cell Lines*. Cell supernatants were centrifuged at 21,000 *g* for 5 min to remove *L. monocytogenes* and other cellular debris. The media supernatants were collected, stored at − 20 °C, and analyzed for released IL-1α by sandwich ELISA according to the manufacturer's instructions (Biosource International, Camarillo, CA). To determine *Listeria*-dependent expression of IL-1α-, S100A8- and S100A9-specific mRNAs, TR146 cell monolayers were washed once with ice-cold Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, Carlsbad, CA) and lysed for mRNA analysis (*see below*).

IL-1α paracrine signaling. *L. monocytogenes* was harvested at log phase, washed, and incubated at MOI 100 with or without 4×10^5 wild-type TR146 cells. As a control for basal cytokine release, fresh culture media without *L. monocytogenes* was incubated with TR146 cells. After 2.5 h, media supernatants were collected and expressed through a 0.22-μm filter syringe to remove *L. monocytogenes* and other cellular debris. The filtered media supernatants were then incubated with naïve wild-type TR146 cells for 24 h. TR146 cell monolayers were washed

once with ice-cold DPBS and lysed for quantification of S100A8- and S100A9-specific mRNAs.

IL-1α dose–response. TR146 and HaCaT cells were seeded 5×10^4 per well into 12-well plates. After overnight culture, monolayers were washed once with sterile DPBS, increasing amounts of recombinant IL-1α (R&D Systems, Minneapolis, MN) were added with or without IL-1Ra (R&D Systems) or BSA control (50 μg ml^{-1} BSA in DPBS) in fresh media and incubated for 24 h. Media was then aspirated, monolayers washed with DPBS, and mRNA was extracted as described below.

Antibiotic protection assay. Bacterial invasion into TR146 cells (2.5×10^4 cells seeded in triplicate 12-well plates) was estimated using an antibiotic protection assay as we have reported previously,[3] except that in most experiments seeding media was replaced after 24 h with fresh media containing BSA control, IL-1α, IL-1α with IL-1Ra or IL-1Ra alone and incubated for an additional 24 h. For the IL-1α/IL-1Ra experimental group, cells were pre-incubated with 100 ng ml^{-1} IL-1Ra for 1 h and then replaced with fresh media containing IL-1α with IL-1Ra. Cells from one triplicate plate were trypsinized for determination of cell number, viability and circumference by trypan blue exclusion for cells in each experimental condition using a Vi-Cell cell viability analyzer (Beckman-Coulter, Fullerton, CA). Cells in a second plate were then inoculated with *L. monocytogenes* at an MOI of 100 in media as described in *Cell Lines*. After 2 h, TR146 cells were washed with DPBS (Mediatech, Manassas, VA) and incubated in fresh medium containing 100 μg ml^{-1} of gentamicin (Sigma-Aldrich, St Louis, MO) for 1.5 h to kill residual extracellular *L. monocytogenes*. Keratinocyte monolayers were then washed with DPBS and osmotically lysed with sterile dH_2O for 15 min. Cell lysates were first mixed with an equal volume of DPBS, diluted 1:10, spiral plated in triplicate (Spiral Biotech, Bethesda, MD) on tryptic soy agar (Becton, Franklin Lakes, NJ), and incubated overnight at 37 °C. CFUs of intracellular *L. monocytogenes* were quantified using a New Brunswick C-110 colony counter (New Brunswick, NJ). RNA from cells in a third replicate plate was collected as described below. Each experiment contained triplicate samples and was repeated a minimum of three times.

RNA collection and quantification. RNA was collected and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) as described by the manufacturer. Complementary DNA was prepared using iScript (Bio-Rad, Hercules, CA) and qRT-PCR was performed using SYBR Green for detection on a Stratagene MX300P (Agilent Technologies, La Jolla, CA). Specific S100A8, S100A9, h-BD2, and IL-1α (150 nM) primers (**Table 1**) were synthesized by Integrated DNA Technologies (Coralville, IA) and used to quantify gene expression normalized to TATA box-binding protein (200 nM). All experiments were conducted with triplicate samples and repeated three times, unless otherwise noted.

Western blot. TR146 cells were washed once with ice-cold DPBS, trypsinized for 5 min and pelleted by centrifugation. Cell pellets resuspended by vortex in RIPA lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxychlorite) with 10% protease inhibitor cocktail (Sigma-Aldrich) for 1 h on ice followed by two freeze-thaw cycles at −80 °C. For collection of samples to examine IL-1RI expression, cells were lysed in the well with RIPA buffer followed by two freeze-thaw cycles. Samples were then centrifuged at 21,000 *g* for 5 min at 4 °C and protein concentrations of the lysis supernatants were determined by BCA (Pierce, Rockford, IL). For western blots, 50 μg total protein was resolved by electrophoresis in a 10% sodium dodecyl sulfate polyacrylamide gel and transferred onto a 0.22-μm nitrocellulose membrane. Blots were blocked with 5% skim milk for 1 h and incubated overnight in 5% skim milk containing anti-S100A8 (1:200), anti-S100A9 (1:1,000), anti-IL-1RI (1:200), or anti-β-actin (1:1,000) antibodies (Santa Cruz Biotech, Santa Cruz, CA). Membranes were then washed in Tris-buffered saline with 1% Tween-20 and probed with the appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit; 1:3,000) for 1 h in 5% skim milk and serially washed in Tris-buffered saline with 1% Tween-20. Pierce ECL western blotting substrate was used for chemiluminescence detection of immunoreactive proteins. Developed films were digitally scanned and analyzed by densitometry for differences in protein expression using ImageJ (National Institute of Health). Densitometric quantification of IL-1RI, S100A8 and S100A9 protein expression was normalized to β-actin using ImageJ and displayed below the immunoblot as fold increase relative to BSA control.

Table 1 Primers used in this study

Gene		Primer
S100A8	Fwd:	5′-GGGCATCATGTTGACCGAGC-3′
	Rev:	5′-GTAACTCAGCTACTCTTTGTGGCTT-3′
S100A9	Fwd:	5′-CGATGACTTGCAAAATGTCGCAG-3′
	Rev:	5′-GCCACTGTGGTCTTAGGGGGT-3′
h-BD2	Fwd:	5′-GACTCAGCTCCTGGTGAAGC-3′
	Rev:	5′-GAGACCACAGGTGCCAATTT-3′
IL-1α	Fwd:	5′-AATGACGCCCTCAATCAAAG-3′
	Rev:	5′-TGGGTATCTCAGGCATCTCC-3′
IL-1RI	Fwd:	5′-ATTGATGTTCGTCCCTGTCC-3′
	Rev:	5′-CCTCCACCTTAGCAGGAACA-3′
TBP	Fwd:	5′-CCACAGCTCTTCCACTCACA-3′
	Rev:	5′-GCAAACCGCTTGGGATTAT-3′

Specific primer sequences for S100A8, S100A9, h-BD2 (human β-defensin 2), IL-1α (interleukin-1α), IL-1RI (interleukin-1 receptor I), and TBP (TATA box-binding protein) used in qRT-PCR analysis.

Statistical analyses. Data are presented as ±standard error of means (s.e.m.) unless otherwise noted. Significant differences between experimental groups were determined using Student's *t*-test. $P<0.05$ was considered to be statistically significant and denoted by asterisk (*).

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DISCLOSURE

The authors declared no conflict of interest.

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