Interferon-γ protects from staphylococcal alpha toxin induced keratinocyte death through apolipoprotein L1

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Abstract

Staphylococcus aureus is a bacterial pathogen that frequently infects the skin, causing lesions and cell destruction through its primary virulence factor, alpha-toxin. Here we show that interferon gamma (IFN-γ) protects human keratinocytes from cell death induced by staphylococcal alpha toxin. We find that IFN-γ prevents alpha toxin binding and reduces expression of the alpha toxin receptor, ADAM10. We determine that the mechanism for IFN-γ mediated resistance to alpha toxin involves the induction of autophagy, a process of cellular adaptation to sublethal damage. We find that IFN-γ potently stimulates activation of the primary autophagy effector, LC3. This process is dependent upon up-regulation of Apolipoprotein L1 (ApoL1). Depletion of ApoL1 by siRNA significantly increases alpha toxin induced lethality and inhibits activation of LC3. We conclude that IFN-γ plays a significant role to protect human keratinocytes from the lethal effects of staphylococcal alpha toxin through ApoL1 induced autophagy.

INTRODUCTION

Staphylococcus aureus infections are frequently observed in the chronic inflammatory skin disease of atopic dermatitis (AD) (Boguniewicz and Leung, 2011; Leung and Guttman-Yassky, 2014). The major virulence factor produced by *S. aureus* is alpha toxin (Bubeck Wardenburg *et al.*, 2007), a pore forming toxin that causes cell lysis (Prevost *et al.*, 2001). In AD skin, a number of cytokines are known to be deregulated (Gittler *et al.*, 2012). The T-helper type 2 (Th2) cytokines IL-4 and IL-13 are over-expressed (Gittler *et al.*, 2012; Hamid *et al.*, 1994; Hamid *et al.*, 1996), resulting in increased susceptibility of keratinocytes to alpha toxin induced cell death (Brauweiler *et al.*, 2014). AD patients prone to *S. aureus* skin infections can also be deficient in IFN-γ production (Machura *et al.*, 2008). However, the role of IFN-γ in protection of primary human keratinocytes from staphylococcal alpha toxin has not been previously explored.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

In monocytes, IFN-γ confers protection from damage caused by bacterial pathogens through a process known as autophagy (Gutierrez *et al.,* 2004). During autophagy, endocytosed bacteria and cellular products are engulfed and isolated in a double-membrane vesicle that is subsequently targeted to the lysosome for elimination (Gutierrez *et al.,* 2004; Levine and Kroemer, 2008). However, the role of IFN-γ induced autophagy in protection of keratinocytes from bacterial pathogens is not known. The induction of autophagy involves a series of sequential steps (Levine and Klionsky, 2004) that lead to activation of light chain 3 (LC3) (Kabeya *et al.,* 2000). LC3 undergoes complex C-terminal proteolytic and lipid modifications, which cause it to translocate from the cytosol to the autophagosomal membrane (Kabeya *et al.,* 2000), targeting the contents for destruction (Levine and Kroemer, 2008).

Although potentially important in pathogen resistance, IFN-γ induced genes that regulate autophagy have not been well described. IFN-γ signaling events culminate in activation of the transcription factor, Signal Transducer and Activator of Transcription 1 (STAT1) (Saha *et al.,* 2010). In the current study, we find that STAT1 potently induces expression of Apolipoprotein L1 (ApoL1), a protein known to be involved in autophagy (Wan *et al.,* 2008). We therefore explored the role of ApoL1 in IFN-γ mediated resistance to alpha toxin induced keratinocyte death and determined its role in mediating autophagy.

RESULTS

IFN-γ protects from alpha toxin induced keratinocyte death

Since cytokine expression has been shown to be deregulated in AD skin, we determined the effects of AD associated cytokines on alpha toxin induced cell death. Primary human keratinocytes were treated with media alone or with IFN-γ, IFN-α, IL-4/IL-13, IL-17, IL-22, or IL-25 for 24 hours. Alpha toxin was then added for an additional 24 hours and cell death was determined by lactic dehydrogenase (LDH) release. We found that primary human keratinocytes were highly sensitive to alpha toxin (Fig. 1a). A striking protection from alpha toxin induced cell-death was induced by pre-treatment with IFN-γ (p< 0.001). Modest protection was also observed with IFN-α. Pre-treatment with the cytokines, IL-17, IL-22, and IL-25 had negligible effects on cellular sensitivity to alpha toxin, while the Th2 cytokines, IL-4/IL-13 increased cell death. Therefore, of the panel of cytokines, IFN-γ was most significantly able to protect against alpha toxin induced lethality.

We then evaluated the dose dependence of IFN-γ on protection from alpha toxin. We determined that IFN-γ induced protection was dose dependent (Fig. 1b), with the maximal protection achieved at 25 ng/ml. Since the downstream events induced by IFN-γ are known to be mediated by the transcription factor STAT1, we next determined the relationship between STAT1 activation and extent of IFN-γ protection from the alpha toxin induced cell death. We found that treatment with 25 ng/ml IFN-γ caused maximal phosphorylation of STAT1 and that the degree of IFN-γ induced STAT1 phosphorylation directly correlated with keratinocyte protection from alpha toxin (Fig. 1c).

IFN-γ mediated protection requires STAT1

We used siRNA directed against STAT1 to determine its role in IFN-γ induced protection from alpha toxin. STAT1 siRNA transfection significantly inhibited STAT1 mRNA expression in keratinocytes (Fig. 2a). Keratinocytes transfected with control siRNA and treated with IFN-γ were shown to be protected from alpha toxin induced cell death (Fig. 2b). In marked contrast, keratinocytes transfected with STAT1 siRNA and treated with IFN-γ became very sensitive to alpha toxin. The increase in cell death induced in STAT1 knockdown cells was significant ($p < 0.001$). We concluded that STAT1 is an essential component in IFN-γ mediated protection of keratinocytes from alpha toxin.

IFN-γ protects from IL-4/IL-13 mediated increases in alpha toxin induced cell death

We recently demonstrated that IL-4 and IL-13 enhance keratinocyte sensitivity to alpha toxin (Brauweiler *et al.*, 2014). Also, previous studies have shown interplay between the Th2 cytokines, IL-4/IL-13, and IFN-γ (Dickensheets and Donnelly, 1999; Galizzi *et al.*, 1988). We were therefore interested in determining whether IFN-γ could block the increased alpha toxin-induced cell death mediated by Th2 cytokines. Primary keratinocytes were treated with IL-4/IL-13 alone and in combination with IFN-γ. As shown in Fig. 3, treatment of keratinocytes with IFN-γ decreased alpha toxin induced cell death. The data in Fig. 3 also demonstrate that IFN-γ could reverse IL-4/IL-13 mediated increases in alpha toxin lethality. A statistically significant decrease in cell death ($p < 0.001$) was observed by co-treatment of IFN-γ with IL-4/IL-13 compared to treatment with IL-4/IL-13 alone.

IFN-γ reduces alpha toxin binding and reduces ADAM10 protein levels

A critical step in alpha toxin induced pore formation is the binding of alpha toxin to the cell surface. After initial attachment, alpha toxin oligerimerizes into a stable heptamer (Song *et al.*, 1996). Since keratinocytes treated with IFN-γ were protected from alpha toxin induced lethality, we thought that it was plausible that IFN-γ treatment reduced alpha toxin binding and heptamer formation. We therefore determined the levels of alpha toxin heptamer bound to keratinocytes that were grown in the presence and absence of IFN-γ. Fig. 4a and b show that cells treated with IFN-γ bound significantly less alpha toxin compared to cells grown in media. In contrast, IL-4/IL-13 treatment had minimal effects on alpha toxin binding (Fig. 4a and b).

Since the amount of heptamer bound to the cell depends on the number of alpha toxin receptors on the cell surface, we examined levels of the alpha toxin receptor, a disintegrin and metalloproteinase 10 (ADAM10) (Wilke and Bubeck Wardenburg, 2010). Keratinocytes were treated with media or IFN-γ for 24 hours, and ADAM10 expression was determined. Fig. 4c and d show that IFN-γ reduced the amount of ADAM10 protein. Prior studies have shown that IL-4/IL-13 treatment did not affect levels of ADAM10 (Brauweiler *et al.*, 2014). In fig. 4e, we examined levels of ADAM10 mRNA. We found that mRNA levels of ADAM10 were unchanged by IFN-γ treatment, suggesting that IFN-γ affected ADAM10 protein turnover, a process reflective of autophagy.

IFN-γ augments alpha toxin induced autophagy

To determine whether IFN-γ induces autophagy in keratinocytes, we assessed LC3 activation. Figs. 5a and b show that alpha toxin itself is a prominent inducer of LC3. LC3 protein levels increased over 3-fold during the 2 hour incubation with alpha toxin. Furthermore, pretreatment with IFN-γ caused further increases in LC3 expression, a process amplified by alpha toxin. We also determined the effects of IL-4/IL-13 treatment on LC3 expression. In contrast to IFN-γ, IL-4/IL-13 did not induce expression of LC3. We found that the increase in LC3 induced by IFN-γ was relatively unaffected by co-treatment with Th2 cytokines (Fig. 5b). In Fig. 5c, we examined localization of LC3 under various cytokine conditions. After 24 hours of cytokine pre-treatment, cells were treated with alpha toxin for 2 hours or remained untreated. Alpha toxin caused a unique re-localization and coalescence of LC3. Notably, LC3 coalescence was not prominent in the cells treated with IFN-γ.

IFN-γ protects from alpha toxin induced keratinocyte death through ApoL1 induced autophagy

To further explore the mechanism for IFN-γ mediated autophagy, we examined a published gene array analysis of keratinocytes treated with IFN-γ (Nograles *et al.*, 2008) to determine genes that were both highly induced by IFN-γ and involved in autophagy. ApoL1 has been previously described to play a role in autophagy (Wan *et al.*, 2008), and we observed a 50-fold increase in ApoL1 expression in IFN-γ treated keratinocytes (Fig. 6a). Furthermore, we demonstrated that IFN-γ mediated induction of ApoL1 was STAT1 dependent, as STAT1 siRNA prevented induction of ApoL1 mRNA by IFN-γ (Fig. 6b), ($p < 0.001$). We also examined the effect of Th2 cytokines on IFN-γ induced ApoL1 expression and found that ApoL1 induction was not significantly affected by co-treatment of IFN-γ with Th2 cytokines (supplemental fig 1).

To determine whether ApoL1 plays a role in mediating protection from alpha toxin induced by IFN-γ, we transfected control and ApoL1 siRNA into primary keratinocytes, and treated the cells with media or IFN-γ. Fig. 6b shows the knockdown of ApoL1. Strikingly, we found that IFN-γ mediated protection from alpha toxin was highly dependent on expression of ApoL1. Fig. 6c shows that IFN-γ was no longer able to protect from alpha toxin in cells transfected with ApoL1 siRNA compared to control siRNA transfected cells ($p < 0.001$). We therefore conclude that ApoL1 plays a critical role in resistance to alpha toxin mediated cell death.

We also examined the consequences of ApoL1 knockdown on autophagy as determined by expression of the marker LC3. In cells treated with control siRNA, significant increases in LC3 expression were observed following treatment with IFN-γ (Fig. 6d and e). Fig. 6d and e also show that the IFN-γ mediated induction of LC3 is ablated in ApoL1 siRNA treated cells ($p < 0.01$). These results demonstrate that IFN-γ mediated induction of ApoL1 plays a critical role in LC3 activation.

DISCUSSION

In this study, we show that IFN-γ protects primary human keratinocytes from staphylococcal alpha toxin induced cell death. We determined that IFN-γ induced resistance to alpha toxin results in activation of the primary effector of autophagy, LC3. We screened a previously published array analysis of IFN-γ treated keratinocytes (Nograles *et al.*, 2008) for genes related to autophagy, and found that IFN-γ significantly induced the autophagy effector, ApoL1. Knockdown of ApoL1 gene expression by siRNA ablated the IFN-γ induced resistance of keratinocytes to alpha toxin. Furthermore, levels of LC3 were severely depleted in keratinocytes transfected with ApoL1 siRNA, demonstrating a crucial role for ApoL1 in autophagy and protection from alpha toxin induced cell death. To our knowledge, this is the first evidence that autophagy and ApoL1 induced by IFN-γ play an essential role in resistance to staphylococcal alpha toxin in human keratinocytes.

The cellular function of ApoL1 is becoming better elucidated. In addition to the role in activation of LC3 demonstrated here, ApoL1 has also been shown to be involved in endocytosis and trafficking to the lysosome, as well as degradation of cellular and pathogenic proteins (Taylor *et al.*, 2014).

To better assess the biological role of autophagy in *S. aureus* infection, mice have been generated which are genetically deficient in the autophagy regulator, Atg16L1 (Maurer *et al.*, 2015), a known activator of LC3. Atg16L deficient mice have significantly increased lethality in response to lung infection induced by *S. aureus*, thus demonstrating a critical biological role for autophagy in resistance to staphylococcal infection.

Interestingly, our data shown here supports the novel concept that alpha toxin itself plays a role in autophagy. Alpha toxin increases LC3 levels, however, it also causes profoundly altered LC3 localization. This change in localization does not occur when cells are protected with IFN-γ. It seems plausible that by altering LC3 localization, alpha toxin may subvert its destruction.

In our studies, we also compared the effects of IFN-γ versus IFN-α in protection from alpha toxin induced cell death. IFN-α, although protective in bronchial epithelial cells (Lizak and Yarovinsky, 2012), was significantly less protective than IFN-γ in keratinocytes (Fig. 1a). Furthermore, IFN-α did not induce ApoL1 expression (supplemental fig 2) to the same extent as IFN-γ. Since epithelial cells robustly respond to IFN-α, it is probable that IFNs have different effects on different cell types.

In addition to functioning as an inducer of autophagy, we find that IFN-γ can antagonize the effects of the Th2 cytokines, IL-4 and IL-13. IFN-γ blocks IL-4/IL-13 mediated increases in alpha toxin induced keratinocyte death. Previous studies have attributed antagonism between IFN-γ and IL-4/IL-13 to effects on gene transcription (Schindler *et al.*, 1995). IFN-γ activates gene transcription through STAT1. In contrast, Th2 effects are mediated through the transcription factor, STAT6 (Travagli *et al.*, 2004). We therefore examined whether IFN-γ could modulate STAT6 induced gene expression. Supplemental Figure 3a shows that expression of a known target of STAT6, Serpin B3 (Sivaprasad *et al.*, 2015; Song *et al.*, 2012), is potently blocked by IFN-γ. However, STAT6 phosphorylation is unaffected by

IFN-γ (Supplemental 3b). Since STAT1 and STAT6 can bind similar target DNA sequences, it has been proposed that STAT1 may function to either block STAT6 binding (Schindler et al., 1995) or interfere with recruitment of critical co-factors (Dickensheets and Donnelly, 1999), thus inhibiting IL-4/IL-13 induced gene transcription. Recent studies have also shown that IFN-γ also induces chromatin remodeling and histone acetylation (Qiao et al., 2013). It remains plausible that altered chromatin structure may also interfere with STAT6 transcription. Since blockade of IL-4/IL-13 function has recently been shown to be a viable treatment for reducing *S. aureus* infection and disease (Beck et al., 2014), a better understanding of the protective effects mediated by IFN-γ may result in potential new therapies to block the effects of Th2 cytokines and improve the outcome of *S. aureus* exacerbated AD.

Each year, in the United States alone, approximately 500,000 infections and 20,000 deaths are caused by *S. aureus* (Klevens et al., 2007). Since alpha toxin is the major staphylococcal virulence factor, it seems plausible that a better knowledge of the factors that modulate alpha toxin induced lethality may help to reduce disease. Our current study demonstrates that IFN-γ treated keratinocytes are resistant to *S. aureus* alpha toxin induced cell death. We find that the resistance induced by IFN-γ is mediated through expression of STAT1 and ApoL1. Further characterization of molecular events that protect from alpha toxin induced lethality will facilitate development of new approaches for treatment of *S. aureus* infections.

MATERIALS AND METHODS

Keratinocyte cell culture

Primary human keratinocytes (Cascade Biologics; Grand Island, NY) were grown in serum-free keratinocyte growth medium (EpiLife; Cascade Biologics), with 1% human keratinocyte growth supplement (Cascade Biologics), 0.06 mM $CaCl_2$, and antibiotics. Cells were grown on collagen matrix coated tissue culture plasticware. IL-4 and IL-13 cytokines were used at 50 ng/ml each. All other cytokines were used at 25ng/ml except when otherwise indicated. All cytokines were from R&D systems (Minneapolis, MN). For LDH assays, keratinocytes were plated at 20,000 per well in a 96 well plate and were allowed to adhere overnight before treatment. Keratinocytes were treated with cytokines or media for 24 hours before treatment with 100 ng/ml alpha toxin (Sigma; St. Louis, MO) for 24 hours. LDH release was determined using the Cyto-Tox One Kit from Promega (Madison, WI) according to the manufacturer's instructions.

Quantitative RT-PCR

Total RNA was isolated using RNeasy Mini Kits (Qiagen, Inc.; Valencia, CA) according to the manufacturer's protocol. One microgram of RNA was reverse transcribed using the Qiagen Quantiscript kit according to manufacturers protocol. RT-PCR was performed and analyzed by the dual-labeled fluorogenic probe method by using an ABI Prism 7300 sequence detector (Applied Biosystems; Foster City, CA). Primers and probes for human Actin, STAT1, Serpin B3, ApoL1 and ADAM10 were purchased from Applied Biosystems. Amplification reactions were performed in MicroAmp optical plates (Applied Biosystems)

in a 25-μL volume as previously described (Howell 2007). All reactions were normalized to beta actin.

siRNA transfection

Second-passage keratinocytes of 50–60% confluence were transfected according to the manufacturer's instructions using Lipofectamine 2000 (Invitrogen) with 20 μM non-targeting, ApoL1, or STAT1 Smartpool siRNA (Dharmacon) in antibiotic free media.

Immunoblot analysis

For analysis of alpha toxin binding and heptamerization, keratinocytes were treated with alpha toxin for two hours, washed with PBS, and harvested in hypotonic lysis buffer with 1% Triton X-100, containing protease inhibitor (Complete, Roche). Cellular debris was pelleted by centrifugation, clarified lysates were resuspended in Laemmli buffer and proteins resolved on a 5–15% gradient gel (Biorad). (The alpha-toxin heptamer is stable during electrophoresis.) Transferred proteins were blotted with anti-alpha toxin antibody (Sigma), and detected by enhanced chemiluminescence (Amersham). Scanned images were quantitated with Image J software. For immunoblotting Actin (Sigma), ADAM10 (Millipore), phospho-STAT1 (Cell Signaling) and LC3 (Abcam), cells were harvested, lysed and probed as described above.

Cell staining and microscopy

Immunofluorescence staining was performed using keratinocytes grown on coverslips. Cells were left untreated or treated with the indicated cytokines for 24 hours prior to addition of alpha toxin for 2 hours. Cells were then washed, fixed with paraformaldehyde and permeabilized briefly with .5% Triton X-100. After blocking with BSA, sections were incubated with primary LC3 antibody (Abcam; Cambridge, MA) for 2 hours at room temperature. Secondary Cy-3 antibody (Jackson labs; West Grove, PA) was added for 1 hour. Images were taken with a Leica Microscope at 40x magnification using SlideBook software.

Statistical analyses

All statistical analysis was conducted using Graph Pad Prism. Comparisons of expression levels were performed using analysis of variance (ANOVA) techniques and Student's *t* tests as appropriate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

|	
AD	Atopic Dermatitis
ADAM10	A Disintegrin and Metalloproteinase 10
ApoL1	Apolipoprotein L1
IL	Interleukin
IFN	Interferon
LC3	Light Chain 3
LDH	Lactate dehydrogenase
RT-PCR	Real Time PCR
STAT1	Signal Transducer and Activator of Transcription 1
Th2	T Helper type 2

References

Beck LA, Thaci D, Hamilton JD, et al. Dupilumab treatment in adults with moderate-to-severe atopic dermatitis. N Engl J Med. 2014; 371:130–9. [PubMed: 25006719]

Boguniewicz M, Leung DY. Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. Immunol Rev. 2011; 242:233–46. [PubMed: 21682749]

Brauweiler AM, Goleva E, Leung DY. Th2 cytokines increase Staphylococcus aureus alpha toxin-induced keratinocyte death through the signal transducer and activator of transcription 6 (STAT6). J Invest Dermatol. 2014; 134:2114–21. [PubMed: 24468745]

Bubeck Wardenburg J, Bae T, Otto M, et al. Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in Staphylococcus aureus pneumonia. Nat Med. 2007; 13:1405–6. [PubMed: 18064027]

Dickensheets HL, Donnelly RP. Inhibition of IL-4-inducible gene expression in human monocytes by type I and type II interferons. J Leukoc Biol. 1999; 65:307–12. [PubMed: 10080532]

Galizzi JP, Cabrillat H, Rousset F, et al. IFN-gamma and prostaglandin E2 inhibit IL-4-induced expression of Fc epsilon R2/CD23 on B lymphocytes through different mechanisms without altering binding of IL-4 to its receptor. J Immunol. 1988; 141:1982–8. [PubMed: 2844892]

Gittler JK, Shemer A, Suarez-Farinas M, et al. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. J Allergy Clin Immunol. 2012; 130:1344–54. [PubMed: 22951056]

Gutierrez MG, Master SS, Singh SB, et al. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell. 2004; 119:753–66. [PubMed: 15607973]

Hamid Q, Boguniewicz M, Leung DY. Differential in situ cytokine gene expression in acute versus chronic atopic dermatitis. J Clin Invest. 1994; 94:870–6. [PubMed: 8040343]

Hamid Q, Naseer T, Minshall EM, et al. In vivo expression of IL-12 and IL-13 in atopic dermatitis. J Allergy Clin Immunol. 1996; 98:225–31. [PubMed: 8765838]

Kabeya Y, Mizushima N, Ueno T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. Embo J. 2000; 19:5720–8. [PubMed: 11060023]

Klevens RM, Morrison MA, Nadle J, et al. Invasive methicillin-resistant Staphylococcus aureus infections in the United States. Jama. 2007; 298:1763–71. [PubMed: 17940231]

Leung DY, Guttman-Yassky E. Deciphering the complexities of atopic dermatitis: shifting paradigms in treatment approaches. J Allergy Clin Immunol. 2014; 134:769–79. [PubMed: 25282559]

Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell. 2004; 6:463–77. [PubMed: 15068787]

Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008; 132:27–42. [PubMed: 18191218]

Lizak M, Yarovinsky TO. Phospholipid scramblase 1 mediates type i interferon-induced protection against staphylococcal alpha-toxin. Cell Host Microbe. 2012; 11:70–80. [PubMed: 22264514]

Machura E, Mazur B, Golemiec E, et al. Staphylococcus aureus skin colonization in atopic dermatitis children is associated with decreased IFN-gamma production by peripheral blood CD4+ and CD8+ T cells. Pediatr Allergy Immunol. 2008; 19:37–45. [PubMed: 17662038]

Maurer K, Reyes-Robles T, Alonzo F 3rd, et al. Autophagy mediates tolerance to Staphylococcus aureus alpha-toxin. Cell Host Microbe. 2015; 17:429–40. [PubMed: 25816775]

Nograles KE, Zaba LC, Guttman-Yassky E, et al. Th17 cytokines interleukin (IL)-17 and IL-22 modulate distinct inflammatory and keratinocyte-response pathways. Br J Dermatol. 2008; 159:1092–102. [PubMed: 18684158]

Prevost G, Mourey L, Colin DA, et al. Staphylococcal pore-forming toxins. Curr Top Microbiol Immunol. 2001; 257:53–83. [PubMed: 11417122]

Qiao Y, Giannopoulou EG, Chan CH, et al. Synergistic activation of inflammatory cytokine genes by interferon-gamma-induced chromatin remodeling and toll-like receptor signaling. Immunity. 2013; 39:454–69. [PubMed: 24012417]

Saha B, Jyothi Prasanna S, Chandrasekar B, et al. Gene modulation and immunoregulatory roles of interferon gamma. Cytokine. 2010; 50:1–14. [PubMed: 20036577]

Schindler U, Wu P, Rothe M, et al. Components of a Stat recognition code: evidence for two layers of molecular selectivity. Immunity. 1995; 2:689–97. [PubMed: 7796300]

Sivaprasad U, Kinker KG, Ericksen MB, et al. SERPINB3/B4 contributes to early inflammation and barrier dysfunction in an experimental murine model of atopic dermatitis. J Invest Dermatol. 2015; 135:160–9. [PubMed: 25111616]

Song KJ, Ahn HJ, Nam HW. Anti-apoptotic effects of SERPIN B3 and B4 via STAT6 activation in macrophages after infection with Toxoplasma gondii. Korean J Parasitol. 2012; 50:1–6. [PubMed: 22451727]

Song L, Hobaugh MR, Shustak C, et al. Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. Science. 1996; 274:1859–66. [PubMed: 8943190]

Taylor HE, Khatua AK, Popik W. The innate immune factor apolipoprotein L1 restricts HIV-1 infection. J Virol. 2014; 88:592–603. [PubMed: 24173214]

Travagli J, Letourneur M, Bertoglio J, et al. STAT6 and Ets-1 form a stable complex that modulates Socs-1 expression by interleukin-4 in keratinocytes. J Biol Chem. 2004; 279:35183–92. [PubMed: 15199062]

Wan G, Zhaorigetu S, Liu Z, et al. Apolipoprotein L1, a novel Bcl-2 homology domain 3-only lipid-binding protein, induces autophagic cell death. J Biol Chem. 2008; 283:21540–9. [PubMed: 18505729]

Wilke GA, Bubeck Wardenburg J. Role of a disintegrin and metalloprotease 10 in Staphylococcus aureus alpha-hemolysin-mediated cellular injury. Proc Natl Acad Sci U S A. 2010; 107:13473–8. [PubMed: 20624979]
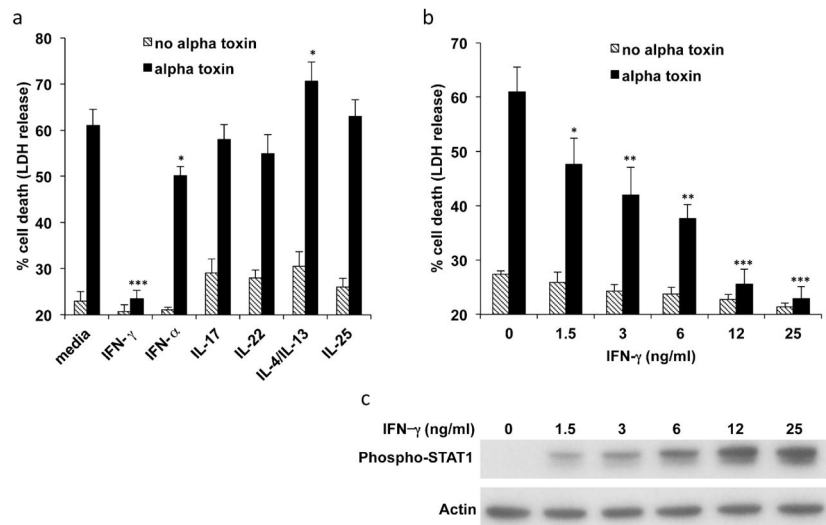
Figure 1. IFN-γ blocks staphylococcal alpha toxin induced keratinocyte death
(a) Primary human keratinocytes were cultured in the presence of media, IFN-γ, IFN-α, IL-17, IL-22, IL-4/IL-13 or IL-25 for 24 hours. 100 ng/ml of alpha toxin was then added, and cell death was measured by LDH release. (b) Keratinocytes were incubated with the indicated concentrations of IFN-γ for 24 hours and then 100 ng/ml alpha toxin was added or (c) cells were harvested and analyzed for expression of phospho-STAT1. Data (a and b) are mean ± SEM, n = 3. *P<0.05; **P<0.01; ***P<0.001 (as compared to the cells grown in medium without cytokines).
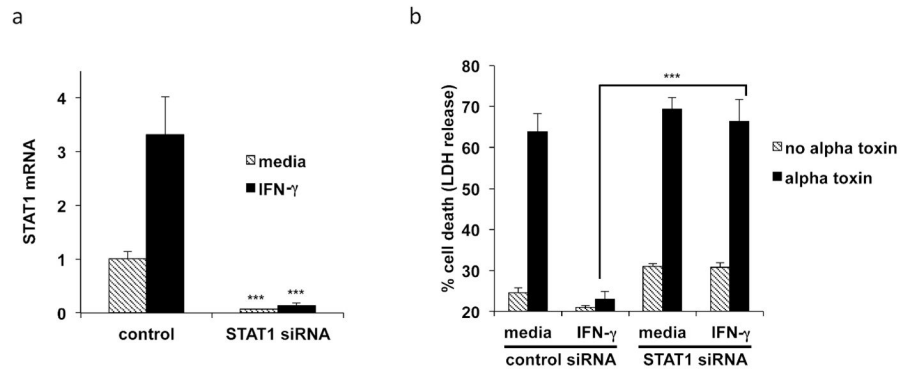
Figure 2. IFN-γ mediated protection from alpha toxin is dependent upon STAT1
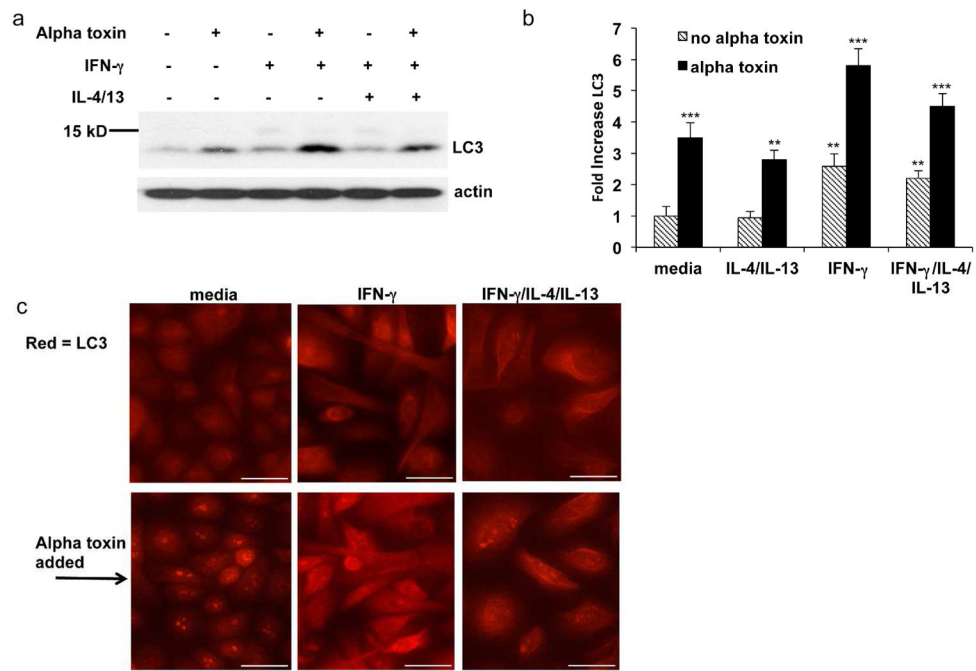(a) Primary keratinocytes were transfected with control (non-targeting) or STAT1 siRNA. Transfected cells were then treated with media or IFN-γ. Gene expression was analyzed by real-time PCR for STAT1. (b) Cells treated as above were subsequently incubated with 100 ng/ml alpha toxin and cell death was measured by LDH release. Data are mean ± SEM, n = 3. ***P<0.001
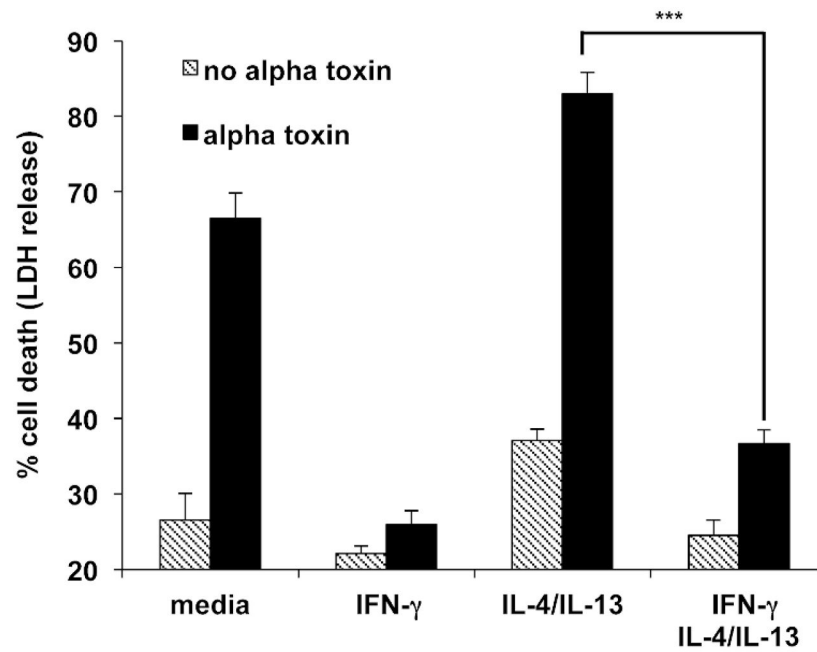
Figure 3. IFN-γ protects from IL-4/13 mediated increases in alpha toxin induced cell death
Primary keratinocytes were treated with media, IFN-γ, IL-4/IL-13 or IFN-γ in combination with IL-4/IL-13. 100 ng/ml alpha toxin was then added for 24 hours, and cell death was measured by LDH release. Data are mean ± SEM, n = 3. ***P<0.001
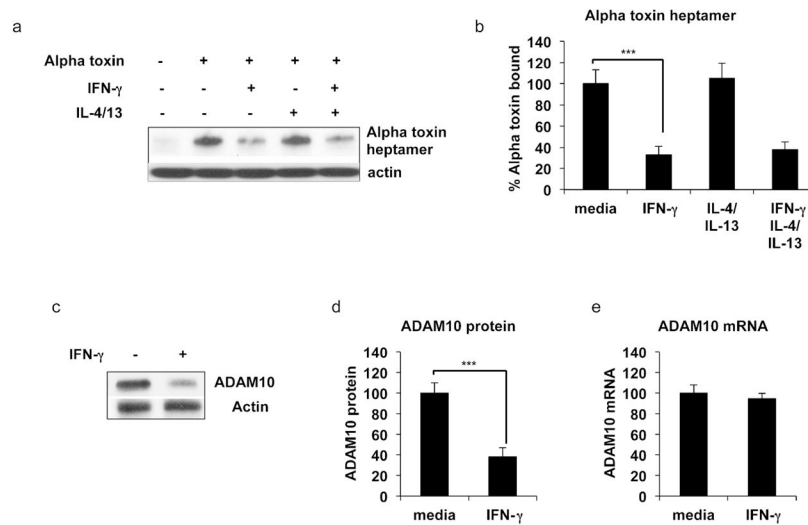
Figure 4. IFN-γ reduces alpha toxin binding to keratinocytes and reduces ADAM10 protein levels
(a) Primary keratinocytes were treated with media, IFN-γ, IL-4/IL-13 or IFN-γ in combination with IL-4/13. 50 ng/ml alpha toxin was then added for 2 hours, and cells were harvested and analyzed by western blot for alpha toxin heptamer formation. (b) Quantitation of alpha toxin binding to keratinocytes under the various cytokine conditions. (c) Cells treated with media or IFN-γ for 24 hours were harvested and analyzed for expression of ADAM10 protein. (d) Quantitation of ADAM10 protein and (e) mRNA levels in the presence and absence of IFN-γ. Data are mean ± SEM, n = 3. ***P<0.001

Figure 5. IFN-γ augments alpha toxin induced autophagy by increasing LC3 levels and modulating LC3 localization

Primary keratinocytes were treated with media or the indicated cytokines for 24 hrs. The indicated cells were then treated with 100 ng/ml alpha toxin for an additional 2 hours. (a) Cells were harvested and levels of the autophagy marker, LC3, were determined by Western blot. (b) Quantitation of LC3 protein levels after pre-treatment with the indicated cytokines followed by further treatment with media or alpha toxin. (c) Immuno-fluorescence microscopy of LC3 protein. Scale bar = 50 μm. Increases in LC3 (b) are mean ± SEM, n = 3. **P<0.01; ***P<0.001 (as compared to the cells grown in medium alone).
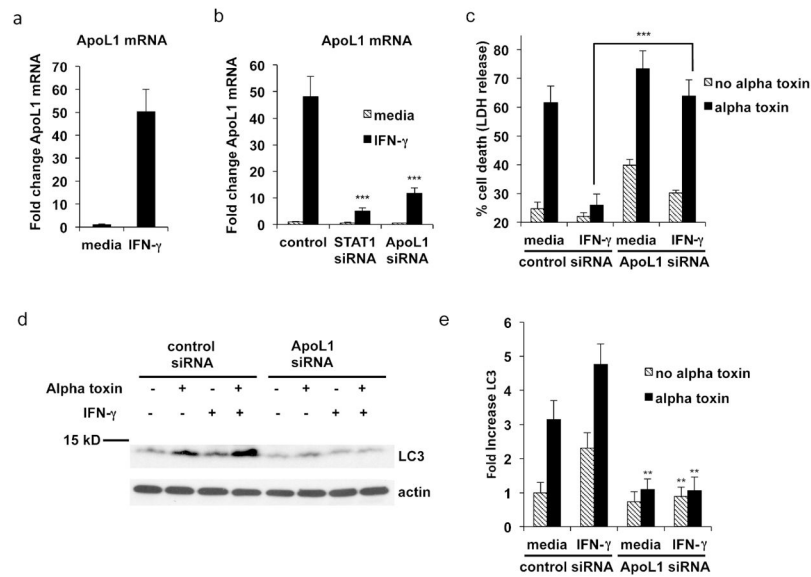
Figure 6. IFN-γ induced ApoL1 mediates autophagy and protects from alpha toxin induced cell death

(a) Primary keratinocytes were treated with media or IFN-γ for 24 hours. ApoL1 gene expression was analyzed by RT-PCR. (b) Primary keratinocytes transfected with control, STAT1 or ApoL1 siRNA were treated with media or IFN-γ for 24 hours. ApoL1 gene expression was analyzed by RT-PCR. (c) Cells transfected with control or ApoL1 siRNA and treated with IFN-γ as described above were subsequently treated with 100ng/ml alpha toxin for 24 hours. Cell death was measured by LDH release. (d) ApoL1 or control transfected cells were treated with media or IFN-γ as indicated and were subsequently treated with alpha toxin for 2 hours. Cells were lysed and LC3 protein expression was analyzed. (e) LC3 protein level quantitation. Data are mean ± SEM, n = 3. **P<0.01; ***P<0.001 (as compared to control siRNA).