Decreased glycogen synthase kinase 3-beta levels and related physiological changes in *Bacillus anthracis* lethal toxin-treated macrophages

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Summary

The lethal factor (LF) component of *Bacillus anthracis* lethal toxin (LeTx) cleaves mitogen activated protein kinase kinases (MAPKKs) in a variety of different cell types, yet only macrophages are rapidly killed by this toxin. The reason for this selective killing is unclear, but suggests other factors may also be involved in LeTx intoxication. In the current study, DNA membrane arrays were used to identify broad changes in macrophage physiology after treatment with LeTx. Expression of genes regulated by MAPKK activity did not change significantly, yet a series of genes under glycogen synthase kinase-3-β (GSK-3β) regulation changed expression following LeTx treatment. Correlating with these transcriptional changes GSK-3β was found to be below detectable levels in toxin-treated cells and an inhibitor of GSK-3β, LiCl, sensitized resistant IC-21 macrophages to LeTx. In addition, zebrafish embryos treated with LeTx showed signs of delayed pigmentation and cardiac hypertrophy; both processes are subject to regulation by GSK-3β. A putative compensatory response to loss of GSK-3β was indicated by differential expression of three motor proteins following toxin treatment and Kif1C, a motor protein involved in sensitivity to LeTx, increased expression in toxin-sensitive cells yet decreased in resistant cells following toxin treatment. Differential expression of microtubule-associating proteins and a decrease in the level of cellular tubulin were detected in LeTx-treated cells, both of which can result from loss of GSK-3β activity. These data provide new information on LeTx's overall influence on macrophage physiology and suggest loss of GSK-3β contributes to cytotoxicity.

Introduction

Bacillus anthracis produces a tripartite bacterial toxin capable of eliciting two toxic effects: oedema and lethality. During intoxication, protective antigen (PA) works in combination with lethal factor (LF) or oedema toxin (EF) to yield lethal toxin (LeTx) or oedema toxin respectively (Brossier and Mock, 2001). Lethal toxin, the focus of this study, causes death in animal models, is toxic to mouse macrophages (Dixon *et al.*, 1999), and provides an advantage to *B. anthracis* during the disease process. As an example, work by Pezard and colleagues indicated LF-negative strains of *B. anthracis* have more than a 1000-fold increase in LD50 over the wild-type strain (Pezard *et al.*, 1991). In addition, many of the signs of late stage anthrax disease can be triggered by LeTx (Dixon *et al.*, 1999). Thus, a better understanding of LeTx cytotoxicity may provide new insights into how *B. anthracis* causes life-threatening disease.

Lethal factor is a zinc-dependent metalloprotease and cleaves mitogen activated protein kinase kinases (MAPKKs) (Duesbery *et al.*, 1998). *In vitro*, LF has been shown to cleave six MAPKKs including MEK(1,2) and MKK(3,4,6,7) (Vitale *et al.*, 2000). These substrates share common amino-terminal sites, which are cleaved by LF. Cleavage by LF may prevent MAPKK interaction with subsequent kinase targets as proteolysis removes an important MAPKK/MAPK docking site. Given the known substrate targets of LF, the toxin has the potential to modulate extracellular signal regulated kinases 1 and 2 (ERK1,2), c-jun N-terminal kinases (JNK) and p38 signalling pathways.

Cleavage of MAPKKs is not likely to be the sole contributing factor to cytotoxicity, given that LF proteolysis

of MAPKKs has been reported in cells resistant to LeTx (Watters *et al.*, 2001) and, at subcytotoxic doses, in cells sensitive to the toxin (Pellizzari *et al.*, 1999). Furthermore, treatment of RAW 264.7 macrophages with inhibitors of the ERK signalling pathways does not cause cell death (our unpubl. obs.), suggesting direct inactivation of this pathway is not the sole contributor to cytotoxicity. Recent studies by Park and colleagues indicated p38 prevents synergistic expression of NF-kappaB genes necessary for antiapoptotic effects in activated macrophages (Park *et al.*, 2002). Collectively, these studies indicate that although cleavage of MAPKK may be part of LeTx activity, a combination of events lead to cell death.

Mouse macrophages from specific genetic backgrounds, such as A/J and C57BL/6 mice, are resistant to LeTx (Friedlander *et al.*, 1993) and provide useful reagents to better understand sensitivity to this toxin. A comparative genetics approach by Watters *et al.* (2001) found that macrophage sensitivity is linked to single nucleotide polymorphisms in the gene encoding for the kinesin-like motor protein Kif1C. Kif1C is also known to localize to the Golgi apparatus and is important for trafficking cargo between this site and the endoplasmic reticulum. In the same study, this group showed that disruption of the Golgi apparatus with brefeldin A results in susceptibility to LeTx in otherwise resistant cells, suggesting localization of Kif1C is important for resistance to the toxin. Whereas the role of Kif1C in LeTx intoxication has not been determined fully, these data further suggest that events outside of direct inactivation of MAPKKs are involved in cytotoxicity.

In an effort to better define LeTx intoxication, in the current study transcriptional profiles were examined from macrophages treated with LeTx. Interestingly, the data revealed distinct alterations in genes subject to direct or indirect regulation by glycogen synthase kinase 3-β (GSK-3β), and notable changes in kinesin motor protein expression. Further analysis revealed an overall loss of GSK-3β protein in LeTx treated cells, thereby explaining the transcriptional profiles. These data emphasize LeTx's capacity to impact multiple cellular pathways leading to cell death.

Results

mRNA profiles in LeTx-treated RAW 264.7 macrophages

DNA array profiling can be used to provide an overall glimpse of cellular activity as it relates to changes in gene expression. In the case of analysing toxin-treated cells, such a profile may reveal disregulation of genes controlled by a particular signalling pathway, providing clues to toxin-activity for further investigation. For these reasons, we
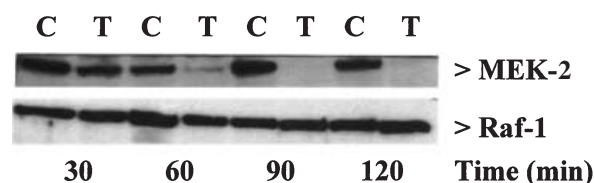

Fig. 1. Immunoblot analysis of proteolytic cleavage of MEK-2 in LeTx-treated RAW 264.7 macrophages. To determine the time-course of LF-mediated cleavage of MEK-2, RAW 264.7 cells were treated with LeTx and extracts were collected at 30 min, 60 min, 90 min and 120 min time-points following treatment. Ten micrograms of extract was resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted using primary antibody reactive to the amino-terminus of MEK-2. Time-points and samples are labelled within the figure. C = control, mock-treated samples, T = LeTx-treated samples. Immunoblot detection of Raf-1 was included to normalize for gel loading and variations in protein abundance.

analysed mRNA changes in RAW 264.7 macrophages following intoxication by LeTx for 30 min or 90 min. As shown in Fig. 1, these are time-points at either 30 min before or 30 min following cleavage of MEK-2. Approximately 4 h following treatment with LeTx, these cells begin to lose viability and show signs of necrotic cell death (data not shown).

The Mouse Atlas™ 1.2 membrane array system, which contains 1185 gene targets that can be grouped into 157 functional categories, was selected for the mRNA analysis. For each time-point, four independent experimental sets were generated for both toxin-treated and mock-treated samples (a total of 16 membranes). Total RNA was extracted from the treated cells, then reverse transcribed, and radiolabelled with [^{33}P]-dCTP. Label incorporation routinely exceeded 90% efficiency. Following the labelling procedure, an equal amount (based on nucleic acid concentration) of probe was hybridized to the membrane arrays. Following this standard treatment, images were captured and spot intensity was quantified.

To identify differentially expressed genes, results from a standard paired *T*-test ($P < 0.05$) were compared to those from an associative analysis ($P < 0.002$). Genes identified by both the standard paired *T*-test and associative analysis are likely real positives, those selected by the standard *T*-test only may contain false positives, and those selected by the associative analysis only are potentially true positives. Candidate genes expressed under only one condition, experimental or control, were not subjected to ratio analysis and were classified as increased or decreased expression. Of the 1185 genes, detectable changes in mRNA levels were found in 108 genes 30 min following toxin treatment. By 90 min, there were 83 differentially expressed genes in the intoxicated macrophages.

Putative signalling pathways targeted by LeTx

Interestingly, analysis of the DNA array data did not reveal a notable change in expression of genes regulated by ERK 1/2 signalling. Of the differentially expressed genes, approximately 15% could be linked to known regulation by ERK 1/2. A similar level of ERK 1/2-regulated genes was detected if a set of genes were selected at random from the array, indicating that toxin treatment did not dramatically change the expression of ERK 1/2 regulated genes. Furthermore, we found that treatment with PD98059, an inhibitor of Raf/MEK interaction and ERK1/2 activation, was not toxic to either LeTx-sensitive or LeTx-resistant cells (data not shown). Finally, p38 and JNK signalling pathways were not active in either control or experimental conditions, as determined by phosphorylation profiles, indicating these LF-targeted pathways did not impact our analyses (data not shown).

These results suggested the inactivation of MAPKKs might not be the sole contributing factor to the changes in physiology of LeTx-treated cells under these experimental conditions. For this reason, the differentially expressed genes were further analysed for other pathways possibly disrupted by LeTx. As summarized in Table 1, several components of the Wnt signalling pathway appeared to be impacted when RAW 264.7 cells were treated with LeTx. Along with alterations in components of the Wnt pathway and genes regulated by Wnt, we also detected changes in genes regulated by GSK-3β. GSK-3β controls levels of β-catenin by phosphorylation, which targets β-catenin for ubiquitinylation and proteasome-mediated destruction. Activation of the Wnt pathway results in GSK-3β inactivation, via dishevelled protein, and subsequent accumulation of β-catenin in the nucleus where transcription is regulated. Thus, it seemed reasonable that the changes in Wnt signalling could be part of a response resulting from inactivation of GSK-3β. Based on these observations, we further analysed the role of the GSK-3β signalling pathway by inactivating this protein with LiCl, as previously described by others (Stambolic *et al.*, 1996; Hedgepeth *et al.*, 1997), before treatment with LeTx. As shown in Fig. 2A, pretreatment of cells with LiCl increases sensitivity to LeTx. RAW 264.7 macrophages pretreated with LiCl exhibited a 10-fold increase in sensitivity to LeTx. In light of these observations, we also determined if LiCl could convert LeTx-resistant cells to a sensitive phenotype. IC-21 macrophages, which are reported to be resistant to LeTx, were pretreated with LiCl and tested for sensitivity to the toxin. As shown in Fig. 2B, these cells became highly sensitive to LeTx following pretreatment with LiCl, further indicating that active GSK-3β promotes cell survival following treatment with LeTx. Finally, treatment with LiCl alone results in minimal cytotoxicity, further suggesting a synergy between loss of GSK-3β activity and other LeTx related activities.

It was unclear whether LiCl pretreatment was potentiating a cytotoxic activity of LeTx or attenuating a protec-

Table 1. Genes differentially expressed in LeTx treated RAW cells associated with Wnt signalling and GSK-3β.

Gene name	Accession #	P value[a]	P' value[a]	Fold[b]	Time-point
Dishevelled, dsh homologue (Drosophila)^	U10115		8.5065E-05	1.85	30 min
Low density lipoprotein receptor^	Z19521		0.00127	1.65	30 min
Discs, large homologue 4 (Drosophila)^	D50621		0.00153	−1.64	30 min
Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain^	U65091	0.03887	4.75066E-18	+	30 min
Matrix metalloproteinase 9 ◇	X72795	0.02438	6.23703E-54	+	30 min
Matrix metalloproteinase 14 ◇	X83536	0.00977	2.40232E-36	+	30 min
Fibroblast growth factor receptor 4 ◇	X59927	0.03683	2.61219E-18	+	30min
Dickkopf homologue 1 (Xenopus laevis)^	AF030433		1.65271E-12	+	30 min
Frizzled homologue 7 (Drosophila)^	U43320		1.53642E-11	+	30 min
Frizzled homologue 6 (Drosophila)^	U43319		7.13486E-77	+	30 min
cAMP responsive element binding protein ⟨	M95106		2.109E-38	−	30 min
Dishevelled 2, dsh homologue (Drosophila)^	U2416		1.44327E-13	3.97	90 min
Catenin (cadherin-associated protein), delta 2^ (neural plakophilin-related arm-repeat protein)	U90331		0.00011	2.04	90 min
Eph receptor B4 ◇	Z49085	0.00951	1.60623E-10	+	90 min
Discs, large homologue 4 (Drosophila) ^	D50621		0.00150	+	90 min
Frizzled homologue 7 (Drosophila)^	U43320		0.00179	+	90 min
Matrix metalloproteinase 2 ◇	M84324	0.00094	2.17779E-07	+	90 min
CCAAT/enhancer binding protein (C/EBP), delta ⟨	X61800	0.01733	3.10031E-16	−	90 min
Dishevelled 3, dsh homologue (Drosophila)^	U41285		1.12474E-07	−	90 min

a. P and P' values represent levels of significance in the standard paired analysis and the associative analysis respectively.
b. Fold refers to the difference in gene expression between control and experimental samples for genes expressed in both conditions (normalized value > 0.48, cf. Methods). Genes expressed only in one condition are represented by + (experimental) or -(control). Genes involved with Wnt signalling pathway are represented by ^. Genes characterized as direct targets of Wnt signalling are represented by ◇. Genes that are considered to be downstream targets of GSK-3β are represented by ⟨.
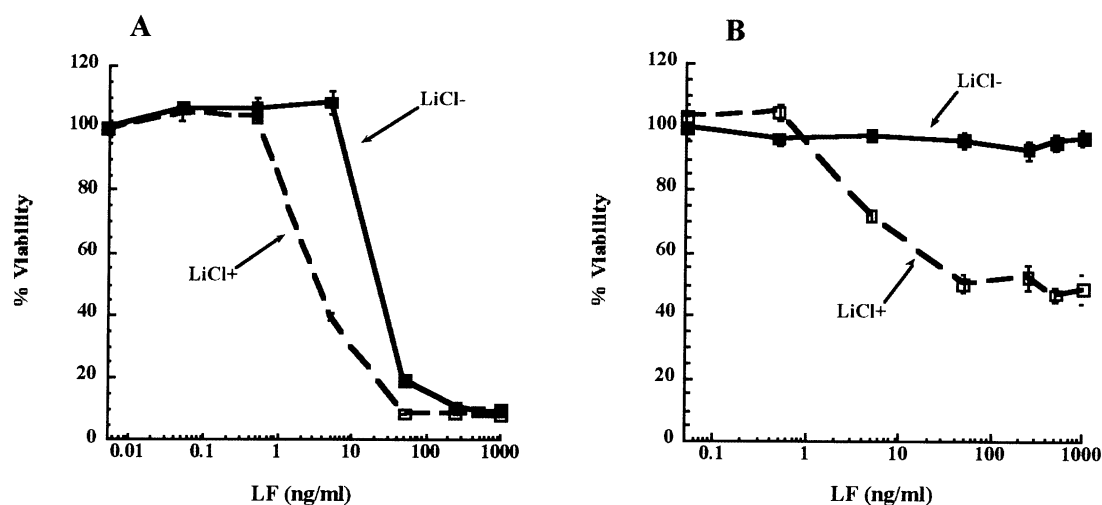
Fig. 2. Relative LeTx sensitivity in macrophages following treatment with LiCl. RAW 264.7 and IC-21 macrophages were pretreated with LiCl and subsequently challenged with LF ranging from 1.0 μg ml^{-1} to 0.005 ng ml^{-1} and a fixed amount of PA (2 μg ml^{-1}). Following a 15 h incubation with LeTx, cells were assayed for viability by CCK-8 staining.
A. Effects of pretreatment with LiCl on LeTx cytotoxicity in RAW 264.7 macrophages.
B. Effects of pretreatment with LiCl on LeTx cytotoxicity in IC-21 macrophages. Solid line = pretreatment with buffer control; dashed line = pretreatment with 20 mM LiCl. Each assay was performed in triplicate and the error bar represents the standard deviation from the mean.

tive response. Furthermore, array data suggested that genes regulated by GSK-3β activity, and unrelated to Wnt signalling, were also modulated in LeTx-treated cells (see Table 1). Additionally, immunoblot analysis of supernatant from LeTx-treated cells did not reveal any detectable Wnt-3 A protein (data not shown), suggesting inactivation of GSK-3β was not caused by increased Wnt expression, and may represent a more global change in GSK-3β. To address this possibility, lysates from LeTx-treated RAW 264.7 macrophages were immunoblotted with GSK-3β antiserum. As shown in Fig. 3A, cells treated with a cytotoxic dose of LeTx showed a marked reduction in the level of detectable GSK-3β. Furthermore, cells treated with a

non-toxic dose of LeTx (5 ng ml^{-1} LF, plus 1 μg ml^{-1} PA) show an approximately 60% decrease in detectable GSK-3β. A modified form of GSK-3β was not detected in the lysates of toxin-treated cells, suggesting a near complete degradation of the protein rather than limited-site cleavage. This possibility was further confirmed by testing the ability of LF to directly cleave GSK-3β in an *in vitro* reaction. As shown in Fig. 3B, co-incubation of LF and GSK-3β did not result in any detectable degradation of GSK-3β. Addition of cell lysates to this reaction did not trigger degradation of GSK-3β (data not shown). Finally, there was no detectable change in the levels of GSK-3β in LeTx-resistant IC-21 macrophages (data not shown),
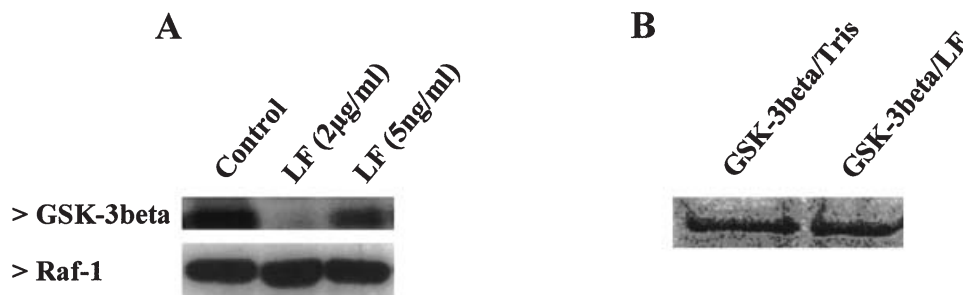

Fig. 3. *In vivo* and *in vitro* analysis of GSK-3β after treatment with LeTx.
A. Immunoblot of GSK-3β from cells treated with LeTx. Lysates were collected 2 h following treatment with LeTx, resolved by SDS-PAGE, and immunoblotted using GSK-3β-specific antibody. Lane 1, buffer control; Lane 2, 2 μg ml^{-1} LF; Lane 3, 5 ng ml^{-1} LF. PA was included at a constant amount of 2 μg ml^{-1}. The corresponding blots and conditions are shown within the figure.
B. *In vitro* stability of GSK-3β in the presence of LF. Purified GSK-3β (2.5 μg) was incubated with 1 μg of LF for 2 h at 37°C, resolved by SDS-PAGE, and stained with Coomassie blue. Immunoblot detection of Raf-1 was included to normalize for gel loading and variations in protein abudance.
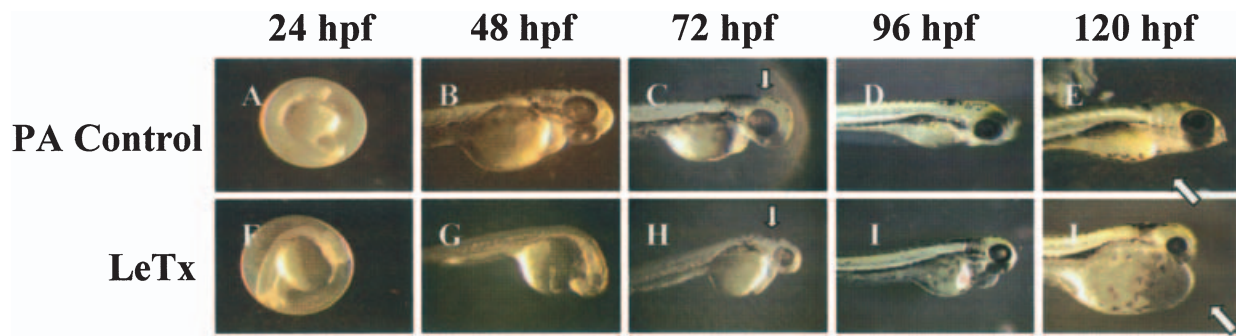
Fig. 4. LeTx treatment of embryonic zebrafish. Zebrafish embryos were treated with LeTx (20 µg ml⁻¹ LF + 20 µg ml⁻¹ PA) between the 4–16 cell stage of development. Control zebrafish were treated with an excess of PA (40 µg ml⁻¹). Lanes A–E, control zebrafish; Lanes F–J, LeTx-treated zebrafish. Arrows indicate points of decreased pigmentation and cardiac hypertrophy following treatment with LeTx.

further suggesting a correlation between decreases in GSK-3β and cytotoxicity.

Effect of LeTx on zebrafish development

The role of GSK-3β signalling in macrophage physiology is poorly defined, and to our knowledge there are no reports of this pathway modulating macrophage activity. GSK-3β activity, via Wnt signalling, has been largely defined in embryonic development. To further address our hypothesis that LeTx is capable of modulating GSK-3β regulated activities, zebrafish embryos were treated with the toxin and analysed for defects in development. Embryos were treated with the toxin at the very early four-cell stage of development. Following treatment, the embryos were examined for defects in embryo development and subsequent growth of the hatched fish. Control embryos were treated with PA alone, LF alone, and PA plus an enzymatically inactive mutant of LF (LF$_{H686C}$). Embryos were treated in triplicate in a 24-well plate (10 embryos/well), for a total of 30 embryos per sample. A dose range of LeTx consisting of 10 µg-40 µg LF/well and a constant amount of PA at 40 µg/well was tested. Amounts of LF in excess of 20 µg were lethal to the embryos (data not shown). Yet, when embryos were treated with functional LeTx at the non-lethal dose, gross defects in development were identified. In contrast, control-treated fish developed with normal phenotypes. A rep-resentative embryo elaborating defects following toxin treatment is shown in Fig. 4. Notably, embryos are slowed in development, demonstrate a delay in pigmentation, and show signs of cardiac hypertrophy when treated with LeTx. As can be seen in Fig. 4J, fish show a major mal-formation of the anterior ventral region. As will be dis-cussed, each of these defects have been linked to Wnt activity in a variety of developmental models. Taken together these data further indicate, in a well-established model, that LeTx is capable of interfering with Wnt related activities, which would occur as a result of the loss of GSK-3β.

Differential expression of kinesin motor proteins

Recent reports suggest a kinesin motor protein, Kif1C, is involved in resistance to LeTx activity (Roberts *et al.*, 1998; Watters *et al.*, 2001). In our analysis, two kinesins, kinesin family member Kif5C and kinesin family member Kif3C, were upregulated, whereas kinesin family member Kif3B was downregulated in toxin treated cells (see Table 2). It has recently been reported that APC and Kif3B proteins co-localize at microtubules along with β-catenin following phosphorylation by GSK-3β (Jimbo *et al.*, 2002). This observation, along with the report of Watters *et al.* (2001), suggest that kinesins not only confer resistance to LeTx, but also respond to LeTx activity by altering transcript levels. If such a hypothesis is true, then we

Table 2. Genes differentially expressed in LeTx-treated RAW cells related to kinesin motor proteins.

Genes names	Accession #	*P* value[a]	*P′* value[a]	Fold[b]	Time-point
Kinesin family member 5C	X61435	0.00615	9.51463E-07	2.47	90 min
Kinesin family member 3C	AF013116	0.00074		1.86	90 min
Kinesin family member 3B	D26077	0.02960	9.98407E-06	−2.26	90 min

a. *P* and *P′* values represent levels of significance in the standard paired analysis and the associative analysis respectively.
b. Fold refers to the difference in gene expression between control and experimental samples for genes expressed in both conditions (normalized value > 0.48, cf. Methods).

Table 3. Real-time PCR analysis of Kif1C in LeTx-resistant and LeTx-sensitive macrophages.

GAPDH	IC-21		RAW	
	[a]C_T	Fold change	[a]C_T	Fold change
Tris	21.4		18.4	
LeTx	20.6		19.8	
Kif1C				
Tris	25.9		26.9	
LeTx	28.4	8.7 decrease	27.1	2.3 increase

a. Mean average of four independent real-time PCR analyses. Fold change was determined by comparison with the standard curve generated for each target gene.

reasoned that resistant versus sensitive cells might show differential expression of Kif1C. Therefore, the level of mRNA encoding for Kif1C was also examined by real-time PCR in IC-21 and RAW 264.7 macrophages following treatment with LeTx. As shown in Table 3, unlike the results from LeTx-treated RAW 264.7 cells, in IC-21 cells there is a decrease in the level of mRNA encoding Kif1C following treatment with LeTx. Kif1C mRNA expression decreased 8.7-fold in IC-21 macrophages, whereas LeTx-sensitive RAW 264.7 macrophages showed a 2.3-fold increase in expression. These data suggest that in addition to single nucleotide polymorphisms in Kif1C between LeTx resistant and sensitive macrophages, the levels of expression in response to the toxin may be a determining factor in cell survival during exposure to the toxin.

Decreased levels of tubulin in LeTx-treated RAW 264.7 macrophages

Microtubules are a major intersecting point for the processes mentioned above. GSK-3β activity and motor protein activity each involve a functional microtubule network (Flaherty *et al.*, 2000; Cui *et al.*, 2002). Furthermore, GSK-3β is reportedly involved in regulating microtubule stability (Sang *et al.*, 2001), and the array data indicated changes in microtubule-interacting proteins. As summarized in Table 4, levels of tubulin beta 4 are reduced 30 min after treatment with LeTx, microtubule-associated protein EB family member 1 (EB1) is reduced by at least 11-fold 90 min following treatment with LeTx, and

microtubule-associated protein 4 is also altered in expression following toxin treatment, making the overall stability of tubulin following treatment with LeTx of interest. As shown in Fig. 5A, following treatment with LeTx, there is a notable decrease in the level of cellular tubulin in RAW 264.7 cells. Densitometry analysis indicated a 60% reduction in total intact tubulin following treatment of the cells with LeTx. This decrease occurs before cell death and cells maintain normal levels of Raf (control) at the time-point of decreased tubulin. Similar to the results with GSK-3β, LF does not appear to directly cleave tubulin. As shown in Fig. 5B there was no detectable loss of tubulin when the protein was incubated with LF.

Discussion

In the current study, we characterize previously unidenti-
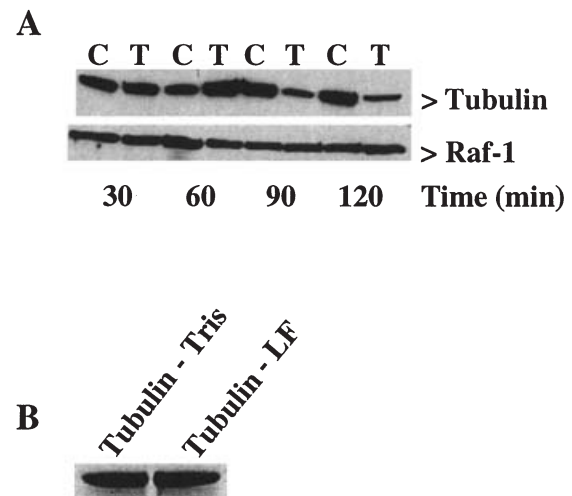
Fig. 5. Changes in cellular tubulin levels following treatment with LeTx.
A. Cell lysates were collected at the indicated time-points from LeTx-treated and mock-treated controls RAW 264.7 macrophages. Extracts were then examined by immunoblot analysis for decreases in total tubulin, in correspondence with cleavage of MEK-2 as shown in Fig. 1. The corresponding conditions are shown within the figure.
B. Stability of alpha-tubulin in the presence of LF. Purified tubulin was incubated with LF for 2.5 h at 37°C, resolved by SDS-PAGE, and stained with Coomassie blue. C = control; mock-treated and T = LeTx-treated samples. Immunoblot detection of Raf-1 was included to normalize for gel loading and variations in protein abudance.

Table 4. Genes differentially expressed in LeTx-treated RAW cells linked to microtubule stability.

Genes names	Accession #	*P* value[a]	*P*′ value[a]	Fold[b]	Time-point
Microtubule-associated protein 4	M72414		8.65563E-11	2.82	30 min
Tubulin, beta 4	M28730	0.00272	2.19067E-11	–	30 min
Microtubule-associated protein, RP/EB family, member 1	U51196	0.00225	4.72168E-38	−11.35	90 min

a. *P* and *P*′ values represent levels of significance in the standard paired analysis and the associative analysis respectively.
b. Fold refers to the difference in gene expression between control and experimental samples for genes expressed in both conditions (normalized value > 0.48, cf. Methods). Genes expressed only in control sample is indicated by (–).

fied changes in macrophage physiology, which occur following intoxication by LeTx. Reportedly, LeTx is cytotoxic to a very limited number of cell lineages, although MAPKKs undergo cleavage by LF in a variety of different cell types. Consequently, yet undefined events outside of the MAPKK signalling pathways may also contribute to LeTx's cytotoxic activity. As a broader approach to analysis of LeTx-intoxicated macrophages, we used a DNA membrane array to evaluate expression of over 1000 categorized genes. The DNA array data was scrutinized with the preconception that most transcriptional changes are the result of disruption of an upstream regulatory pathway, or part of a compensatory response by the cell. Thus, this data is used as a guide to find LeTx-induced disturbances at the protein level. We believe our data reflects results of both pathway disruption (e.g. GSK-3β) and compensatory responses (e.g. kinesin motor proteins). Using this perspective, analysis of the expression data suggested the Wnt signalling pathway could be disrupted in LeTx-treated macrophages and this lead to the observation of GSK-3β loss in these cells. Our DNA array analysis also indicated that kinesin motor-protein expression and tubulin stability may be altered in LeTx-treated macrophages.

The first indication of perturbations in a GSK-3β-regulated signalling pathway came from our observation that transcriptional changes occurred in proteins involved in Wnt signalling, as well as genes regulated by this pathway. We also detected changes in a group of genes regulated by GSK-3β outside of the Wnt pathway. This observation suggested these changes might be due to an overall loss of GSK-3β activity after treating cells with LeTx. In line with this, results from the LiCl inhibitor studies suggest GSK-3β is necessary for survival of LeTx-treated cells and loss of the protein is not an inconsequential downstream event in intoxication.

A plausible explanation for the observed effects would link LiCl inactivation to GSK-3β inhibition of NF-κB-regulated expression of factors that are important for cell survival. A report by Hoeflich *et al*. (2000) found that NF-κB is regulated by GSK-3β within transcriptional complexes. Thus, inactivation of GSK-3β by LiCl could block NF-κB transcriptional regulation. Park *et al*. (2002) have recently shown that NF-κB synergizes with p38 to regulate survival genes in the presence of inflammatory stimuli and that LeTx, by inactivating the p38 pathway, promotes cell death. Thus, blocking GSK-3β function by LiCl treatment may heighten sensitivity to LeTx by preventing the expression of factors that promote survival. It must be noted however, that LiCl treatment may be having a broad effect on cell signalling; thereby impacting targets outside of the GSK-3β pathway. For this reason the LiCl sensitization data should be considered predominantly in light of results from the zebrafish, immunoblot, and DNA array experiments. In fact, detailed analysis of the DNA array data

does further support the putative loss of GSK-3β in LeTx-treated cells. β-catenin, AP-1, cyclic AMP-response element binding protein, NF-κB, Myc, heat shock factor-1, nuclear factor of activated T-cells and CCAAT/enhancer-binding proteins are known targets of GSK-3β regulation and are represented on the membrane arrays. Of these, β-catenin, cyclic AMP-response element binding protein and CCAAT/enhancer-binding protein show changes in expression following treatment of macrophages with LeTx.

It seems likely that GSK-3β signalling is most important at the low, apoptosis-inducing, levels of LeTx treatment. LiCl treatment did not alter the high-dose effects of LeTx, but enhanced cytotoxicity at low-doses of the toxin. Furthermore, cell death in IC-21 macrophages occurred at a relatively slow rate, indicative of apoptosis, which would be in agreement with the finding of Popov *et al*. (2002). Collectively, these data indicate that necrotic cell death occurs even in the absence of inhibition of GSK-3β, but the low-dose effects of the toxin are enhanced in cells in which GSK-3β has been attenuated.

Wnt signalling, which is regulated by GSK-3β, plays a major role in various stages of embryonic development. Thus, we used a developing zebrafish model to further confirm LeTx's impact on this pathway. Phenotypic changes, indicative of defects in Wnt signalling, were revealed in toxin-treated embryos. Temporal loss of pigmentation and cardiac hypertrophy were the two prominent phenotypes of LeTx-treated embryos. Both of these developmental processes have been shown to involve regulation by Wnt. Dorsky *et al*. (2000) recently reported on the requirement of Wnt in regulating pigment cell formation in zebrafish. Furthermore, Wnt signalling is known to be involved with cardiogenesis and modulation of cardiac hypertrophy via GSK-3β-related activities (Hardt and Sadoshima, 2002). Thus, the resulting phenotypes from LeTx-treated embryos correspond with the developmental defects expected following Wnt inactivation. Given a possible role for Wnt in sensitizing macrophages to LeTx by inactivating GSK3-β, cells may be more susceptible to the toxin at anatomical sites containing Wnt.

In an important study by Watters and colleagues, a kinesin-like motor protein, Kif1C, was implicated in cellular resistance to LeTx (Watters *et al*., 2001). Therefore, changes in kinesin expression, as determined by the DNA arrays, were of immediate interest. In this study, we present further evidence to implicate motor proteins in the accommodation of LeTx activity. Our experiments examined the role of motor proteins from a perspective different from Watters and colleagues which identified the resistance loci based on single nucleotide polymorphisms in the *kif1C* gene. Yet, there are correlations between these two studies. Watters and colleagues also found that destabilizing the cellular localization of Kif1C with brefeldin-A converts resistant cells to a LeTx-sensitive phenotype.

Thus, alterations in Kif1C's ability to function correctly may be linked to LeTx-sensitivity. In the absence of the ability to make these adjustments, cells would be rendered more susceptible to the toxin, as is the case with RAW 264.7 macrophages.

An encompassing model for the observed effects can be proposed from these findings and centres around the loss of GSK-3β. In such a case, loss of GSK-3β and subsequent alterations in synergy with NF-κB may render the cell incapable of promoting survival. Within the same model, loss of GSK-3β could lead to alterations in microtubule stability and corresponding compensatory responses involving kinesin protein expression. Defining specific details of such a model will require continued investigation. Furthermore, such a model should not dismiss a role for inactivation of MAPKKs as a contributing factor. The instigating event leading to loss of GSK-3β is not clear and could be a downstream result of MAPKKs inactivation. However, this cannot be the full explanation for cell death because other cells with inactivated MAPK signalling do not necessarily succumb to the toxin. In total, results from this current study furthers our appreciation of LeTx as a virulence factor with multifaceted activities and should encourage the continued investigation of this toxin's ability to impact multiple cellular events.

Experimental procedures

Cell culture and viability assay

Cell culture media and additives were purchased from Invitrogen. The cell lines RAW 264.7 (BALB/c mouse macrophage derived Abelson leukaemia virus induced tumour macrophages) and IC-21 (C57BL/6 mouse macrophage derived SV-40 transformed peritoneal macrophages) were obtained from the American Type Culture Collection and maintained in RP-10 medium supplemented with 10% fetal bovine serum. The cell lines were grown at 37°C in a humidified atmosphere of 6% CO_2. Cell viability was determined by visual observation for cell rounding and quantified using the Cell Counting Kit-8 (CCK-8), which determines cell viability via detection of cell dehydrogenase reduction of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt]. Percent viability was determined using the formula $100*(A450_{test} - A450_{background})/(A450_{control} - A450_{background})$, wherein test = toxin-treated samples, control = buffer alone and background = untreated cells. Viability assays were performed in triplicate.

Purification of recombinant PA and LF

Recombinant forms of PA and LF were purified from *E. coli*/BL-21 (DE3) (Novagen) using His-tagged affinity chromatography according to the manufacturer's protocol. Purified proteins were desalted, passed over a polymyxin B agarose column, and eluted according to manufacturer's instructions (Sigma). Purified proteins were assayed for endotoxin using the Limulus Amebocyte Lysate (LAL) kit supplied by Biowhittaker which has a detection minimum of 0.03 endotoxin units ml^{-1}.

LeTx-treatment of RAW 264.7 macrophages for DNA array analysis

For mRNA profiling, RAW 264.7 cells were grown in 75 cm^2 tissue culture-treated flasks as a confluent monolayer (approximately 1×10^7 cells per flask) and treated with 2 μg ml^{-1} LF and 2 μg ml^{-1} PA in a volume of 10 ml. RNA and protein were isolated from cells 30 min and 90 min following treatment with LeTx. Control cells were treated with an equal volume of endotoxin-free 20 mM Tris, pH 8.0 and subjected to the same incubation times.

RNA isolation and cDNA probe synthesis

Total RNA was isolated using Trizol according to manufacturer's instruction (Invitrogen Life Technologies). After the initial extraction, RNA samples were subjected to treatment with DNase (1 U) for 30 min. This reaction was terminated by the addition of 1 ml of Trizol at which point the samples underwent a second round of extraction. The final RNA samples were solubilized in RNase-free water and immediately processed for cDNA synthesis. The yield of total RNA for each sample was determined spectrophotometrically (OD_{260}) and the quality of RNA was confirmed by analysis on a 0.6% formaldehyde agarose gel. Approximately 25 μg of total RNA was used for the synthesis of each cDNA probe. For each sample, an initial annealing reaction was carried out in a 23 μl volume wherein RNA was combined with 1 μl of dATP, 1 μl of dTTP, 1 μl of dGTP (10 mM stocks), 2 μl primer mix (Clontech), 6 μl 5 × First Strand Buffer (Invitrogen Life Technologies), and 2 μl RNase-free water (Ambion) and incubated at 90°C for 2 min and 42°C for 20 min. At the completion of the annealing reaction, 2 μl [^{33}P]-dCTP (10 mCi ml^{-1}; New England Nuclear), 3 μl 0.1 M DTT, 1 μl SuperScript II reverse transcriptase (200 U μl^{-1}), and 1 μl (40 U μl^{-1}) Ribonuclease inhibitor (Invitrogen Life Technologies) was added to the reaction and the sample incubated an additional 2 h at 42°C. Finally, each probe reaction was incubated at 94°C for 5 min in the presence of 5 μl 10 × denaturation solution (1 M NaOH, 10 mM EDTA) and 50 μl 2 × neutralization solution (1 M NaH_2PO_4, pH 7.0). NucAway spin columns (Ambion) were used according to manufacturer's instructions to remove unincorporated nucleotides.

Hybridization, image captures and data analysis

Each Mouse Atlas™ 1.2 expression array underwent a prehybridization reaction by incubating with 70 μl heat-denatured salmon sperm DNA (10.0 mg ml^{-1}) and 5.0 ml ExpressHyb solution (Clontech) at 68°C for 2.0 h. The purified cDNA probe was then hybridized to the Atlas™ array membrane at 68°C for 18 h. Following hybridization, membranes were washed twice with 50.0 ml 2 × SSC, 1.0% SDS for 20 min, followed by two washes with 50.0 ml 0.1 × SSC, 0.5% SDS for 20 min. The arrays were then exposed to a storage phosphor screen (Molecular Dynamics-Amersham Pharmacia Biotech) for ~48 h. Images were acquired by scanning on a Storm phosphorimager (Molecular Dynamics-Amersham Pharmacia Biotech) and intensities were quantified with ArrayVision software (Incyte Genomics). Data analysis was

performed using an associative analysis approach as previously described by Dozmorov and Centola (2003).

Analysis of MEK-2, tubulin, Raf-1 and GSK-3β

Protein extracts were taken from the phenolic phase of a Trizol extraction (Invitrogen Life Technologies) of RAW 264.7 and IC-21 cells treated with LeTx or control as described in the above section. The protein samples were solubilized with 1% SDS and the relative concentrations were determined by the Bronsted-Lowry method (Lowry *et al.*, 1951). For each sample subjected to analysis of MEK-2, tubulin, and Raf-1, 10 μg of protein was separated by SDS-PAGE and then electro-transferred to a poly-vinylidene difluoride (PVDF) membrane. For GSK-3β analysis, 20 μg of protein was used. Immunoblots were subjected to incubation with primary antibody (sc-524; Santa Cruz Biotechnology) (1:500 dilution) targeting the amino-terminus of MEK-2, primary antibody against alpha-tubulin (CP06; Oncogene research product) (1:10 000 dilution), primary antibody against Raf-1 (SC-7198; Santa Cruz Biotech) 1:1000 dilution, or primary antibody specific for the carboxy-terminus of GSK-3β (361528; Calbiochem) (1:1000 dilution). Primary antibodies were detected with the corresponding horseradish peroxidase-linked secondary antibodies. Blots were developed with the ECL chemiluminescence detection system according to the manufacturer's protocol (Amersham Pharmacia Biotech). Relative levels of tubulin and GSK-3β were determined by densitometry using NIH Image V. 1.62 software.

The ability of LF to mediate direct cleavage of tubulin and GSK-3β was analysed using purified tubulin from bovine brain (Molecular Probes) and purified GSK-3β (Upstate) in combination with LF. Cleavage of tubulin by LF was assayed by incubating 5 μg of tubulin with 1 μg of LF or Tris buffer control at 37°C for 2.5 h and resolving with SDS-PAGE. Similarly, the stability of GSK-3β was examined by incubating 2.5 μg of GSK-3β with 1 μg of LF or Tris buffer control at 37°C for 2 h and resolving with SDS-PAGE.

Zebrafish maintenance and LeTx treatment

Zebrafish (*Danio rerio*) were maintained at 28.5°C on a 14 h light/10 h dark cycle. Embryos were collected and maintained at 28.5°C and the stage of development was determined by morphology and reported as hours post fertilization (hpf). Fish embryos were placed in a 24-well plate with 10 fish ml^{-1} well^{-1} and incubated with 20 μg ml^{-1} LF and 20 μg ml^{-1} PA and were observed for seven days post treatment for morphological changes.

Inactivation of GSK-3β

To implicate GSK-3β signalling in LeTx-mediated cytotoxicity, RAW macrophages were treated with 20 mM LiCl for 2 h. Cells were subsequently treated with a range of LF (1.0 μg ml^{-1} to 0.005 ng ml^{-1}) plus PA (2.0 μg ml^{-1}) or 20 mM Tris as a buffer control. Cell viability, as determined by CCK-8 assay, was compared between LeTx-treated cells in the presence and absence of LiCl verses the corresponding Tris buffer controls.

Semi-quantitative real-time PCR

Real-time PCR was performed on total RNA extracted from LeTx-treated and mock-treated macrophages. Three primer sets were designed for Kif1C using Primer 3 software (Rozen and Skaletsky, 2000). All primers were 21 bases in length. Pilot experiments showed optimal reaction with the primer set covering a region starting at nucleotide 732 and ending at nucleotide 832 in the open reading frame of *kif1C* and this pair was used throughout the real-time PCR analysis. A similar approach was used for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference sample as well and optimal primers were used covering a region starting at nucleotide 764 and ending at nucleotide 872. In each reaction, 500 ng of total RNA was reverse transcribed using the TaqMan reverse transcription kit (Applied Biosystems) according to manufacturer's instructions. Product from the reverse transcription was used as template in a subsequent real-time PCR. Each 25 μl reaction contained 400 nM primer, 0.5 ng of template, and 12.5 μl 2× SYBR-green PCR master mix (Applied Biosystems) which includes dNTPs and AmpliTaq gold polymerase. The cycle protocol for real-time PCR was as follows: 1 cycle at 95°C for 10 min; 40 cycles of 95°C for 15 s, 48°C for 30 s, 60°C for 30 s, and a final cycle of 60°C for 10 min. These real-time PCR reactions and detection of accumulated product were performed in a Cepheid Smart-Cycler (Cepheid) and data was analysed using the accompaning software. Relative levels of expression were determined based on calculations involving the critical threshold (C_T) value of each sample, which was considered to be the point of greatest change in SYBR-green fluorescence along the curve. Calculations of fold changes in mRNA levels were made using the C_T value in a standard curve approach according to manufacturer's instructions (Applied Biosystems). All samples and reactions were performed in quadruplicate.

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