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A Novel Role for TGF-Beta Signaling in Epileptogenesis

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A Novel Role for TGF-Beta Signaling in Epileptogenesis

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University of California, Berkeley

Committee in charge:

Professor Daniela Kaufer, Chair

Professor John Ngai

Professor Darlene Francis

Professor Donald Rio

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Abstract

A Novel Role for TGF-Beta Signaling in Epileptogenesis

By

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Doctor of Philosophy in Neuroscience

University of California, Berkeley

Professor Daniela Kaufer, Chair

Epilepsy, one of the most common neurological disorders, affects between 0.5 and 2 percent of the population worldwide. Post-traumatic epilepsy is one of the most difficult forms of epilepsy to treat and the mechanism leading to the characteristic hypersynchronous activity has yet to be elucidated. Previous clinical studies have shown that perturbations in the blood-brain barrier seen after brain injury may be associated with epileptic activity in these patients. We previously demonstrated that albumin is critical in the generation of epilepsy following blood-brain barrier compromise and in this thesis TGF-β pathway activation is identified as the underlying mechanism.

We demonstrate that direct activation of the TGF-β pathway by TGF-β1 results in epileptiform activity similar to that following exposure to albumin. Co-immunoprecipitation revealed binding of albumin to TGF-β receptor II and Smad2 phosphorylation confirmed downstream activation of this pathway. Transcriptome profiling demonstrated similar expression patterns following BBB breakdown, albumin and TGF-β1 exposure, including modulation of genes associated with the TGF-β pathway, early astrocytic activation, inflammation, and reduced inhibitory transmission. Importantly, TGF-β pathway blockers suppressed most albumin-induced transcriptional changes and prevented the generation of epileptiform activity. Microarray data also revealed changes in many astrocytic genes following BBB disruption and albumin treatment including downregulation of glutamate transporters, glutamine synthetase, the potassium channel Kcnj10, and several connexins. Primary cortical cultures enriched for astrocytes were treated with albumin and confirmed these changes in gene expression, indicating a disruption in astrocytic glutamate and potassium buffering. Finally cell type specific changes in TGF-β signaling pathways were evaluated with primary cortical cultures enriched for astrocytes or neurons. In astrocytes, treatment with albumin resulted in preferential activation of the canonical TGF-β pathway mediated by the TGF-β type I receptor Alk5. Treatment resulted in an increase in Smad2 phosphorylation at 4 hours and an increase in Smad1 phosphorylation as well as Alk5 expression at 24 hours. In neurons, albumin treatment resulted in preferential activation of an alternate TGF-β pathway

mediated by the TGF-β type I receptor Alk1. Treatment resulted in an increase in Smad1 phosphorylation at 4 and 24 hours as well as a small increase in Alk1 expression at 24 hours. In addition, TGF-βR2 expression was decreased in both cell types and TGF-β pathway blockers prevented astrocytic Smad2 phosphorylation. Our present data identifies the TGF-β pathway as a novel putative epileptogenic signaling cascade and therapeutic target for the prevention of injury-induced epilepsy.

Acknowledgements

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Chapter 1 – INTRODUCTION

Blood-brain barrier disruption in posttraumatic epilepsy

Epilepsy, affecting 0.5-2% of the population worldwide, is one of the most common neurological disorders. Traumatic brain injury (TBI) is known to be a major predisposing factor for neocortical epilepsy accounting for approximately 20% of symptomatic epilepsies and 5% of all epilepsies (Hauser, Annegers et al. 1991). Seizures may occur immediately after TBI, which are likely due to the trauma itself or may develop over months and even years. Post-traumatic epilepsy refers to the occurrence of seizures at least a week after TBI (Pitkanen and McIntosh 2006). While immediate seizures can be prevented using antiepileptic drugs (Temkin, Dikmen et al. 1990), there is currently no way to prevent the development of post-traumatic epilepsy (Garga and Lowenstein 2006).

Epileptiform activity can be induced in experimental animals by early life trauma resulting in cortical malformations (Jacobs, Gutnick et al. 1996), by focal application of epileptic agents, such as penicillin (Prince and Wilder 1967; Opdam, Federico et al. 2002), or by chronic injury to, or deafferentation of, the adult cerebral cortex (Halpern, Purpura et al. 1972; Prince and Tseng 1993). In most of these models (similar to the situation in people), a period of days to weeks is required for the development of epileptic activity (Prince and Tseng 1993; Hoffman, Salin et al. 1994). This “critical time window” (Graber and Prince 2004) is referred to as the period of "*epileptogenesis*". While the characteristic electrical activity in the epileptic cortex has been extensively studied, the mechanisms underlying epileptogenesis are poorly understood.

Following injury, local compromise of blood-brain barrier (BBB) integrity is common (Tomkins, Kaufer et al. 2001; Neuwelt 2004; Abbott, Ronnback et al. 2006; Oby and Janigro 2006). The blood-brain barrier, formed by endothelial cells lining cerebral microvessels, serves to protect the central nervous system and maintain a constant microenvironment by regulating the passage of molecules from the blood into and out of the brain extracellular space (Risau and Wolburg 1990). Ultrastructural studies on human epileptic tissue show clear BBB abnormalities, including increased micropinocytosis and fewer mitochondria in endothelial cells, a thickening of the basal membrane, and the presence of abnormal tight junctions (Kasantikul, Brown et al. 1983; Cornford and Oldendorf 1986; Cornford 1999). These studies have raised the possibility that vascular damage, and specifically BBB opening, may serve as a trigger event leading to epilepsy. This hypothesis has been confirmed by animal studies, in which opening of the BBB (induced by focal application of bile salts) was sufficient to induce delayed epileptiform activity four days following treatment (Figure 1; (Seiffert, Dreier et al. 2004; Ivens, Kaufer et al. 2007).

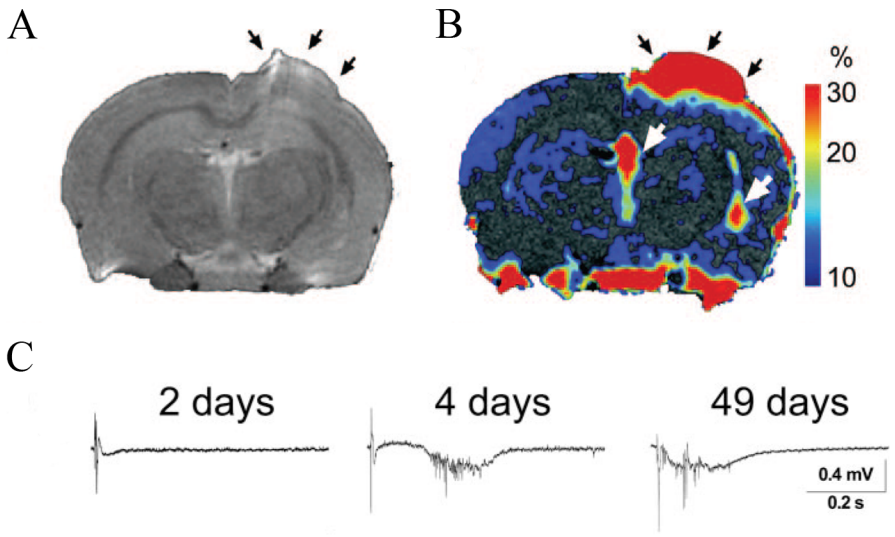


Figure 1: Focal BBB disruption causes prominent cortical dysfunction. **A,**T2 sequence MRI of a rat brain 24 h following BBB opening. Note local brain swelling due to vasogenic edema in the treated region (arrows). **B,** Colour-coded T1 image showing areas of significant signal change after gadolinium-DTPA injection. Colour bar represents percentage of contrast enhancement. White arrows point to the intraventricular choroid plexus, normally lacking a BBB. **C,** Electrophysiological recordings performed in slices from BBB-treated cortical regions from operated animals. Paroxysmal events were first recorded in slices 4 d after treatment. Figure modified from Ivens et al., 2007 and Seiffert et al., 2004.

Subsequent studies have shown that albumin, the most common serum protein, is sufficient to recapitulate the epileptiform activity induced by BBB disruption. Treating animals with serum, denatured serum or aCSF containing albumin all induced epileptiform activity. In addition, albumin treatment increased the number of GFAP-stained cells, indicating a prominent astrocytic response (Seiffert, Dreier et al. 2004) and other studies using human and rat epileptic tissue also found changes in glial properties (Pollen and Trachtenberg 1970; Bordey and Sontheimer 1998; Hinterkeuser, Schroder et al. 2000; Jauch, Windmuller et al. 2002; Eid, Lee et al. 2005). When FITC-labeled albumin was applied to cortical slices, albumin uptake was seen in cells which expressed GFAP (marker for asrocytes) but was not observed in cells expressing MAP2 (neuronal marker). Furthermore, a competition assay where non-labeled albumin was increased in the presence of FITC-albumin demonstrated that albumin uptake is receptor-mediated (Ivens, Kaufer et al. 2007). Since the type 2 TGF-β receptor has been shown to bind albumin in lung endothelial cells (Siddiqui, Siddiqui et al. 2004), a TGF-β receptor antagonist was applied which reduced the number of FITC-albumin positive cells in slices and also blocked epileptiform activity when the antagonist was used *in vivo* (Ivens, Kaufer et al. 2007).

Therefore, it appears that albumin acts on astrocytes via its interactions with TGF-β receptors as illustrated in the proposed model in figure 2. The objective of this thesis was to determine in detail the role of albumin and TGF-β receptors in the mechanism underlying epileptogenesis following BBB compromise. A better understanding of this mechanism may enable the identification of a therapeutic target and development of targeted drugs which are able to block epileptic foci with minimal effects on normal brain function. The goal of such a treatment is to prevent the network alterations causing hypersynchronous activity rather than suppressing the abnormal activity once it has been generated.

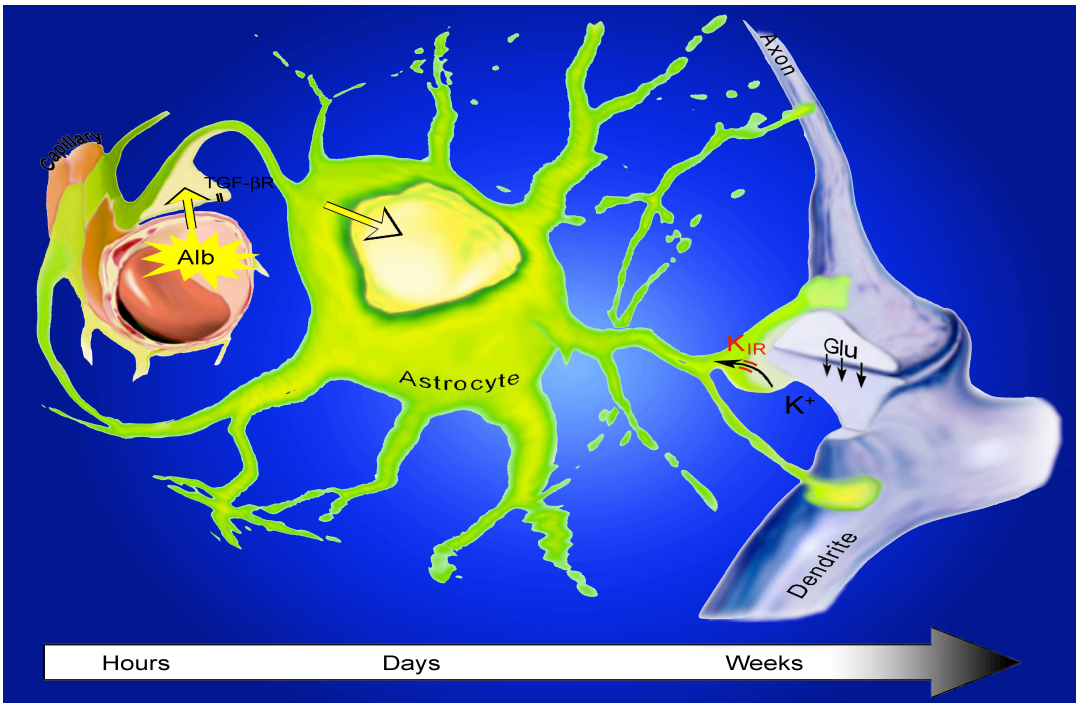


Figure 2: Proposed model for neocortical epileptogenesis. Picture by Dominik Zumsteg.

Chapter 2 – Transcriptome profiling reveals TGF-β signaling involvement in albumin-induced epileptogenesis.

INTRODUCTION

Uptake of serum components such as albumin and IgGs, associated with BBB disruption, has been demonstrated in various cell populations. Albumin is taken up by astrocytes (Ivens, Kaufer et al. 2007; van Vliet, da Costa Araujo et al. 2007), neurons (Marchi, Angelov et al. 2007; van Vliet, da Costa Araujo et al. 2007)**,** and microglia although to a lesser extent (van Vliet, da Costa Araujo et al. 2007), while IgG uptake has been found in neurons (Rigau, Morin et al. 2007). In rat lung endothelial cells, albumin endocytosis is mediated by transforming growth factor beta receptors (TGF-βRs), leading to phosphorylation of the proximate effector of the canonical TGF-β signaling pathway, Smad2, and translocation of the activated Smad2/Smad4 complex to the nucleus (Siddiqui, Siddiqui et al. 2004). TGF-βRs are also implicated in albumin uptake by astrocytes, as blocking TGF-βRs prevents albumin uptake and suppresses albumin-induced epileptiform activity (Ivens, Kaufer et al. 2007). This raises the possibility that albumin activation of the TGF-β signaling pathway serves as the underlying mechanism, however, this hypothesis remains unconfirmed.

Here we show that activation of the TGF-β signaling pathway is sufficient to induce epileptiform activity. Furthermore, we show that global transcriptional cascades induced by TGF-β1 or albumin exposure before the development of epileptiform activity (during the epileptogenesis window) are nearly identical and can be blocked by application of TGF-βR blockers. Given the pleiotropic effects of the TGF-β signaling pathway, these findings provide a plausible mechanism for epileptogenesis following brain injury, and advocate a specific therapeutic target.

RESULTS

TGF-β signaling is sufficient to induce epileptiform activity

To assess the hypothesis that activation of the TGF-β signaling pathway is the mechanism underlying albumin-induced epileptogenesis, this pathway was directly activated by incubating neocortical slices with TGF-β1 (10 ng/mL) in artificial cerebrospinal fluid (aCSF) and electrophysiological recordings were performed. These recordings were compared to those of slices treated with a solution containing serum levels of electrolytes and 0.1mM albumin (aSERUM, previously shown to induce epileptogenesis (Ivens, Kaufer et al. 2007), albumin in aCSF, or aCSF (control). Spontaneous, prolonged and hypersynchronous interictal-like activity was observed in slices treated with aSERUM after 6-10 hours (n = 6 out of 9 slices, 3 animals) but never in aCSF treated slices (Fig. 1B). When albumin was added to the control aCSF solution, epileptiform activity was recorded only in response to stimulation of the white matter (Fig. 3C; n = 8 out of 12 slices, 6 animals).

Importantly, epileptiform activity was also recorded following incubation in TGF-β1, which is similar to that seen following treatment with aSERUM and albumin in aCSF (Fig. 3C; n = 5 out of 5 slices, 4 animals; n = 7 out of 9 slices, 3 animals; and n = 8 out of 12 slices, 6 animals, respectively). Recordings were performed 4-12 hr following treatment. While treatment with either TGF-β1 or albumin in aCSF resulted in the appearance of evoked epileptiform activity, only the altered electrolytic solution (i.e. aSERUM) resulted in spontaneous activity in the slice preparation. Importantly, when aSERUM was applied without albumin neither spontaneous or evoked epileptiform activity was recorded. In all three cases (albumin, TGF-β1, and aSERUM), the evoked epileptiform activity was all-or-none in nature, paroxysmal, prolonged and propagating along the cortical slice, similar to that seen following BBB opening with bile salts (Seiffert, Dreier et al. 2004; Ivens, Kaufer et al. 2007) and typical to that observed in acute models of epilepsy (Gutnick, Connors et al. 1982). No epileptiform activity was seen in the control aCSF treated slices.

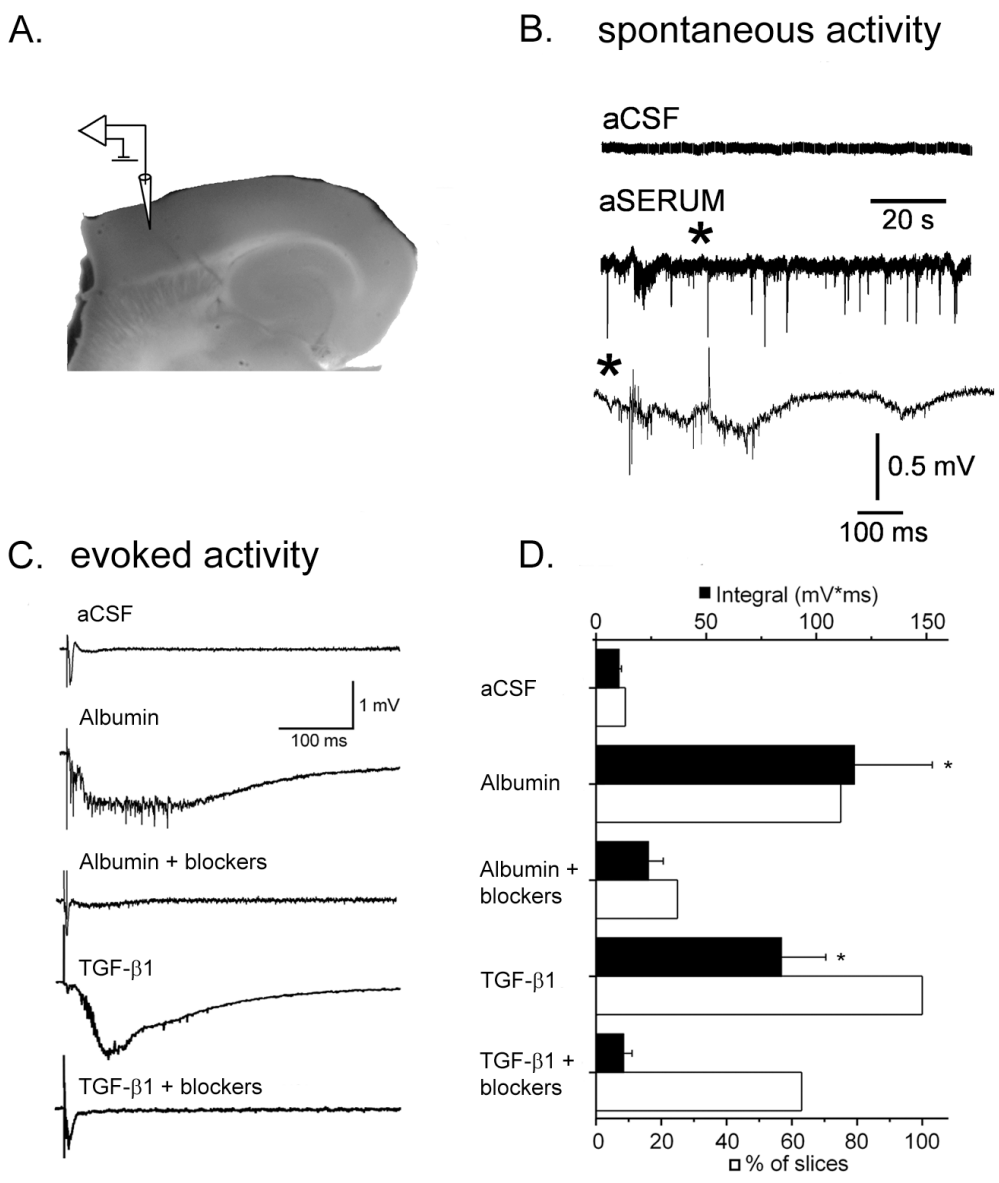


Figure 3. TGF-β signaling induces epileptiform activity.
A, Photograph of a brain slice displaying electrode positioning. The stimulating electrode was placed at the white-gray matter border. **B,** Extracellular recordings showing spontaneous interictal-like epileptiform activity following treatment with artificial serum containing albumin (aSERUM). Asterisk refers to the region corresponding to the slower time scale shown in the lower trace. **C,** Evoked responses from slices treated with aCSF, albumin, albumin plus TGF-β receptor blockers, TGF-β1, or TGF-β1 plus TGF-β receptor blockers. TGF-β receptor blockers prevent epileptiform activity induced by albumin or TGF-β1 treatment. **D**, Comparison of mean event integral (black bars) in the 50-500 ms time range (post stimulation) shows a significant increase in the integral of the delayed epileptiform field potential in the albumin and TGF-β1 treated slices but not in slices treated with TGF-β receptor blockers. The white bars represent the percentage of slices with paroxysmal, epileptiform activity. Error bars indicate s.e.m. Asterisks indicate $p<0.05$.

To further confirm that the TGF-β1 induced epileptiform activity was dependent on the TGF-βR mediated pathway, additional trials of the above experiments were performed in the presence of two TGF-βR blockers (SB431542 and anti-TGF-βRII antibody). TGF-βR blockers prevented epileptiform activity induced by TGF-β1 or albumin (Fig. 3C). The measured integral of the field potential (albumin: 117.2 ± 35.4 mV*ms; TGF-β1: 84.1 ± 20.1 mV*ms) was significantly lower in slices treated with albumin or TGF-β1 in the presence of TGF-βR blockers (albumin and blockers: 23.7 ± 6.9 mV*ms, n = 20 slices, 4 animals, p = 0.001; TGF-β1 and blockers: 12.5 ± 3.9 mV*ms, n =8 slices, 4 animals, p =0.005) (Fig. 3D).

Albumin binds TGF-βRs and activates the TGF-β pathway

To determine whether albumin binds to TGF-β receptors, co-immunoprecipitation using antibodies against albumin or TGF-βRII was performed on cortical lysate samples (obtained from naïve rats). An expected band corresponding to TGF-βRII was detected in samples immunoprecipitated with the TGF-βRII antibody. More importantly, this band was also detected in samples pre-incubated with albumin when immunoprecipitated with the albumin antibody and probed for TGF-βRII (Fig. 4A). These results reveal a direct interaction between albumin and TGF-βRII. In the canonical TGF-β signaling pathway, Smad2 and/or 3 are phosphorylated following TGF-β receptor activation and form a complex with Smad4, which then translocates into the nucleus and activates transcription (Beattie, Stellwagen et al. 2002). To investigate whether albumin activates downstream components of the TGF-β pathway, Smad2 phosphorylation levels in cortical lysates were assessed by Western blot, revealing an increase in Smad2 phosphorylation during the epileptogenic time window in animals exposed to albumin as compared to sham-operated controls (Fig. 4B).

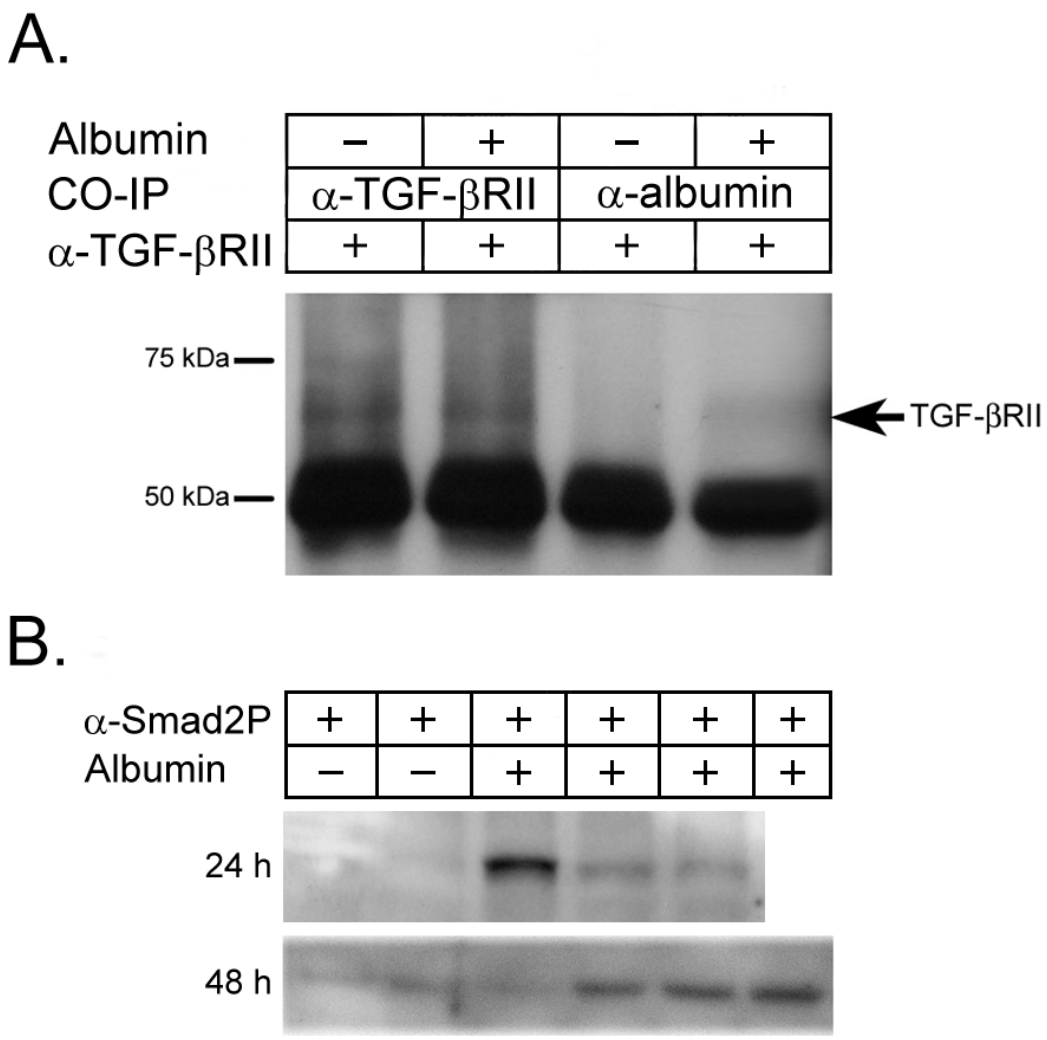
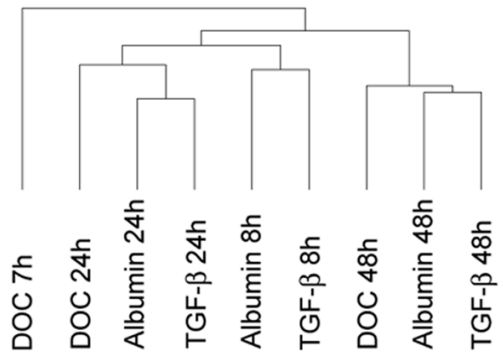


Figure 4. Albumin activates the TGF-β pathway. **A,** Albumin and TGF-βRII immunoprecipitations. Samples treated or untreated with serum albumin were co-immunoprecipitated with antibodies directed against albumin or the TGF-βRII receptor. All samples were then probed with an anti-TGF-βRII antibody. The band at 50kDa is the heavy chain of the precipitating antibody. **B,** Western blot analysis of Smad2-P 24 and 48h following albumin treatment. Each band represents a different animal (24 hours: controls n = 2, albumin n = 3; 48 hours: controls n = 2, albumin n = 4).

Similar transcriptional profiles follow BBB opening, albumin and TGF-β1 treatments

BBB opening or exposure to albumin *in vivo* (Ivens, Kaufer et al. 2007), as well as *in vitro* exposure of neocortical slices to albumin or TGF-β1 (Fig. 3C) all result in the gradual development of hypersynchronous neuronal epileptiform activity. The delayed appearance of abnormal activity (5-7 hours *in vitro* and >4 days *in vivo* (Ivens, Kaufer et al. 2007)) suggests a transcription-mediated mechanism. In search of a common pathway and transcriptional activation pattern that underlie epileptogenesis following BBB opening, transcriptome analysis was performed using Affymetrix rat microarrays. RNA was extracted from cortical regions of rats treated with sodium deoxycholate (DOC, to induce BBB opening), albumin or TGF-β1 for various durations (7/8, 24, 48hr). These time points were chosen in order to evaluate changes in transcription occurring before the appearance of epileptiform activity. Control RNA was extracted from cortical regions excised from sham-operated animals. Hierarchical clustering analysis of these arrays showed that overall the three treatments resulted in strikingly similar gene expression profiles, as arrays representing similar time points clustered together regardless of the treatment (Fig. 5A). These similarities are exemplified in figure 5B, which shows a high correlation between the expression profiles for the albumin and TGF-β1 treatments at 24 hours ($r^2 = 0.75$, $p < 0.0001$).

A.



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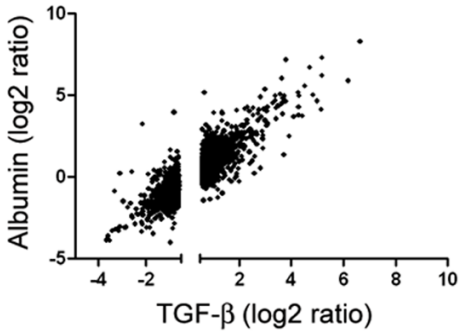

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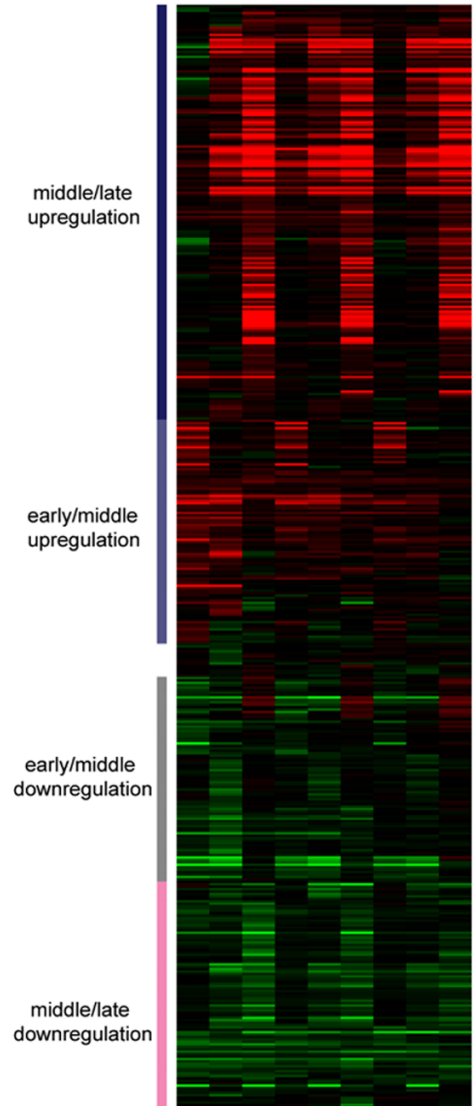


GO Term	# genes	%	p-value
early/middle downregulation			
protein metabolic process	21	23.1	4.4E-04
catalytic activity	28	30.8	1.3E-03
nucleotide binding	13	14.3	1.5E-02
intracellular signaling cascade	10	11.0	1.6E-02
response to wounding	6	6.6	1.6E-02
response to metal ion	3	3.3	2.0E-02
calcium ion binding	7	7.7	3.5E-02
sugar binding	4	4.4	3.9E-02
early/middle upregulation			
cell differentiation	14	18.4	4.2E-04
response to external stimulus	8	10.5	4.2E-03
protein kinase cascade	6	7.9	4.9E-03
cellular component organization & biogenesis	15	19.7	5.5E-03
growth	5	6.6	1.1E-02
apoptosis	7	9.2	1.4E-02
metabolic process	28	36.8	3.4E-02
cellular localization	7	9.2	3.8E-02
regulation of signal transduction	5	6.6	4.6E-02
middle/late downregulation			
synaptic transmission	13	10.7	1.7E-07
protein binding	41	33.9	1.0E-04
transporter activity	17	14.1	3.0E-04
regulated secretory pathway	5	4.1	1.2E-03
intracellular transport	10	8.3	3.0E-03
ion transport	10	8.3	6.1E-03
glutamate signaling pathway	3	2.5	6.8E-03
extracellular structure organization & biogenesis	4	3.3	1.0E-02
regulation of lipid metabolic process	3	2.5	1.6E-02
nucleotide binding	16	13.2	2.7E-02
response to hormone stimulus	4	3.3	4.0E-02
regulation of growth	4	3.3	4.3E-02
middle/late upregulation			
cell proliferation	21	12.1	5.2E-06
protein binding	64	36.8	9.3E-06
cell cycle	17	9.8	1.1E-05
response to stress	23	13.2	7.9E-05
enzyme regulator activity	14	8.1	1.8E-03
DNA replication	7	4.0	4.2E-03
positive regulation of angiogenesis	3	1.7	7.4E-03
intracellular signaling cascade	21	12.1	7.5E-03
cell differentiation	24	13.8	9.8E-03
nitric oxide metabolic process	3	1.7	1.3E-02
protein metabolic process	39	22.4	1.6E-02
cytoskeleton organization & biogenesis	10	5.8	1.9E-02
response to extracellular stimulus	5	2.9	2.2E-02
immune system process	13	7.5	2.6E-02

Figure 5. Genome-wide transcriptional analysis following epileptogenic treatments. **A,** Hierarchical clustering of arrays corresponding to 7/8, 24 and 48 hours following DOC, albumin and TGF-β1 treatments. Note how arrays cluster together for each time point across all treatments. Genes showing at least a 1.5 fold change in expression were included. **B,** Linear regression analysis between TGF-β1 and albumin treatments at 24 hours. Only genes with a fold change equal to or greater than 1.5 for the TGF-β1 treatment were included. **C,** Hierarchical cluster analysis of genes showing correlation (> 0.95) between all treatments. Selected clusters were annotated with DAVID to reveal biological themes common to all treatments. Color bar indicates range of $\log_2$ ratios.

To identify biological themes common to the three treatments, the gene list was filtered to include genes showing at least a 1.5 fold change in expression and a Pearson correlation coefficient ≥ 0.95 for pair-wise comparisons between all treatments (see methods). Hierarchical clustering was performed and the main clusters were used for gene ontology (GO) analysis with DAVID (Database for Annotation, Visualization, and Integrated Discovery)(Dennis, Sherman et al. 2003). DAVID calculates the probability that particular GO annotations are overrepresented in a given gene list using a Fisher exact probability test. Molecular function and biological process GO terms with a p-value < 0.05 containing at least three genes were considered significant. This analysis revealed major gene expression trends that occur in response to all three epileptogenic treatments (Fig 5C). Early responses include genes involved in general stress-related cellular, metabolic and intracellular signaling pathways; early responses persisting to later time points include inflammatory processes as well as genes involved in induction of cell cycle, differentiation, proliferation, and apoptosis; responses at middle to late time points include repression of synaptic transmission and ion transport genes (Fig 5C; for complete GO term annotation results see Supplementary Tables 1-4).

Gene level expression profiles

Selected GO term groups were chosen for further analysis of individual gene expression profiles (Fig. 6). The most dramatic change observed in all treatments across all time points was the early and persistent upregulation of genes associated with immune response activation (Fig. 6A-B). Inflammatory genes included NF-kappa B pathway related genes, cytokines and chemokines (*Il6, Ccl2, Ccl7*), transcription factor *Stat3*, the pattern recognition receptor *CD14* and extracellular matrix proteins (*Fn1* and *Spp1*) (Fig. 6A). Activation of the complement pathway was also prominent (Fig. 4B) and included C1 subcomponents (*C1qa, C1qb, C1qg*), the associated protease *C1s, Masp1*, and *C2*. A significant neuronal response was prominent in the middle-late time points and included downregulation of genes associated with GABAergic (inhibitory) neurotransmission (including the GABA A receptor subunits, *Gabra4, Gabrd, Gabrg1*, and *Gabrb2* as well as glutamic acid decarboxylase (*Gad67*) (Fig. 6C) and modulation of genes associated with glutamatergic (excitatory) neurotransmission (including upregulation of the ionotropic glutamate receptor subunits, GluRdelta2 and GluR1 and downregulation of the NMDA receptor subunits, *NR2B, NR2A* and *NR2C* and the metabotropic glutamate receptor,

mGluR7, Fig. 6D). Furthermore, a variety of voltage gated ion channels including calcium, sodium, chloride, and potassium channels were affected by all three epileptogenic treatments (Fig. 6E), including a noteworthy downregulation of voltage gated (*Kv7.3* and *Kv8.1*) and inward rectifying (*Kir3.1*) potassium channels. We also found significant modulation of glial-specific genes beginning at the early time point (Fig. 6F): the cytoskeletal proteins *GFAP* and vimentin (*Vim*), and several calcium binding proteins (*S100a6, S100a10, s100a11*) were all upregulated while gap junction connexins 30 and 43 (*Cx30* and *Cx43*) and the inward rectifying potassium channel *Kir4.1* were downregulated. Microarray-based gene expression measurements for selected genes were further verified using quantitative real-time PCR. Expression patterns were similar although the magnitude of the fold changes sometimes differed (Supplementary Fig. 1)

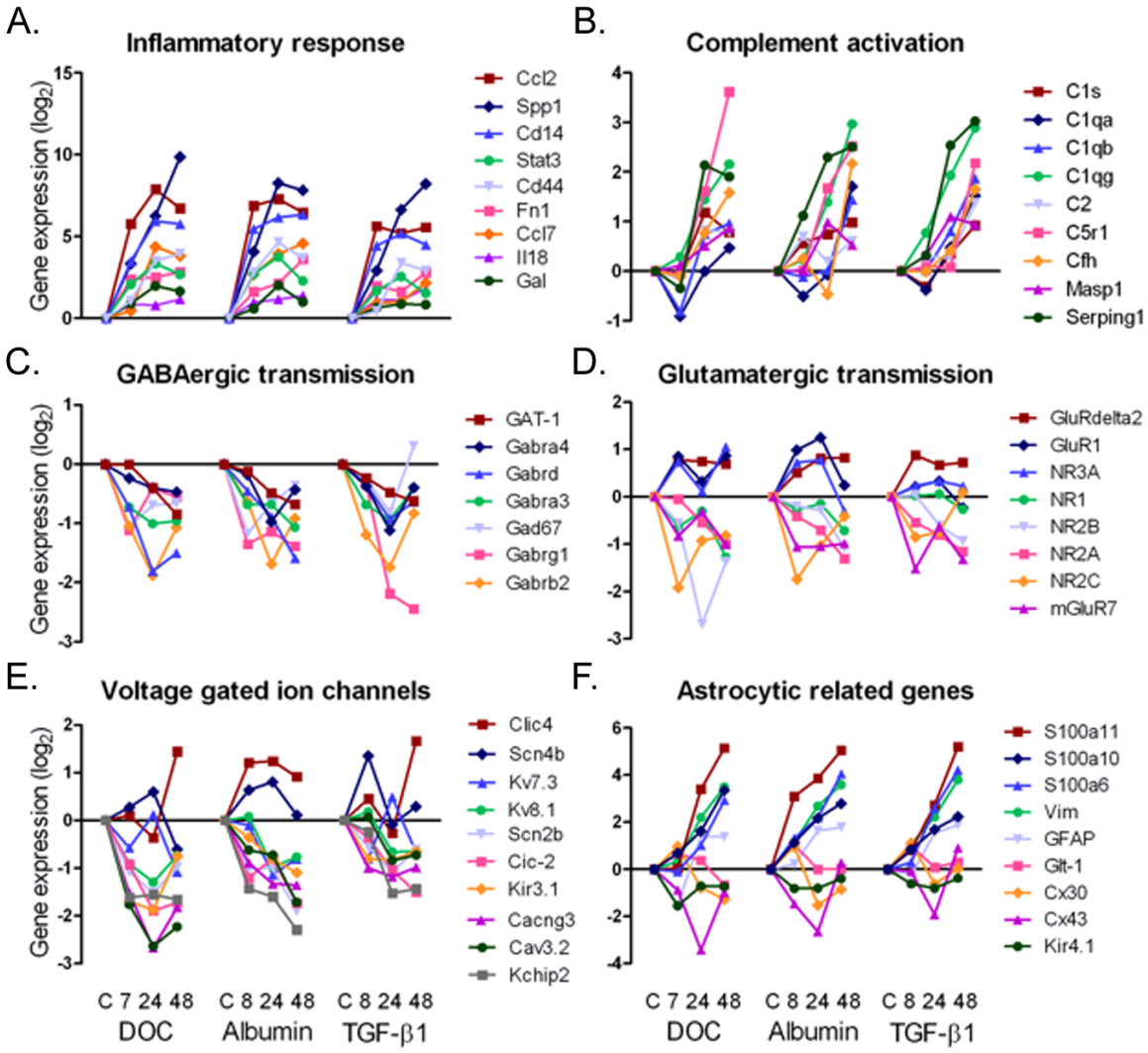


Figure 6. Gene ontology annotation analysis. Log_2 ratios for selected genes from GO annotation analysis involved in (**A**) inflammation (**B**) complement activation (**C**) GABAergic transmission (**D**) glutamatergic transmission. (**E**) Astrocytic-related genes. Numbers below data points correspond to the various treatments (7/8, 24, and 48 hours).

TGF-β pathway activation underlies epileptogenic transcriptional response

Given the high correlation between expression profiles following the three epileptogenic treatments, combined with the biochemical evidence that albumin binds to TGF-β receptors and the physiological evidence that TGF-β1 induces evoked epileptiform activity, the extent to which each treatment activates transcription of genes known to be associated with the TGF-β signaling pathway was assessed using GenMAPP (Salomonis, Hanspers et al. 2007). 43% of genes analyzed in the TGF-β signaling pathway were modulated by both treatment with albumin and TGF-β1. Genes which showed at least a 1.5 fold change in expression following albumin or TGF-β1 treatment, are highlighted in figure 7. Importantly, 86% of genes modulated by TGF-β1 treatment are also modulated following albumin treatment indicating a high degree of overlap. To check the specificity of this pathway activation, additional pathways were analyzed. Indeed, there was still a high degree of overlap but the percent of genes modulated by both albumin and TGF-β1 treatments was much lower. For example, for the androgen receptor and alpha6-beta4 integrin signaling pathways, only 21.7% and 24% of genes analyzed were modulated by both treatments.

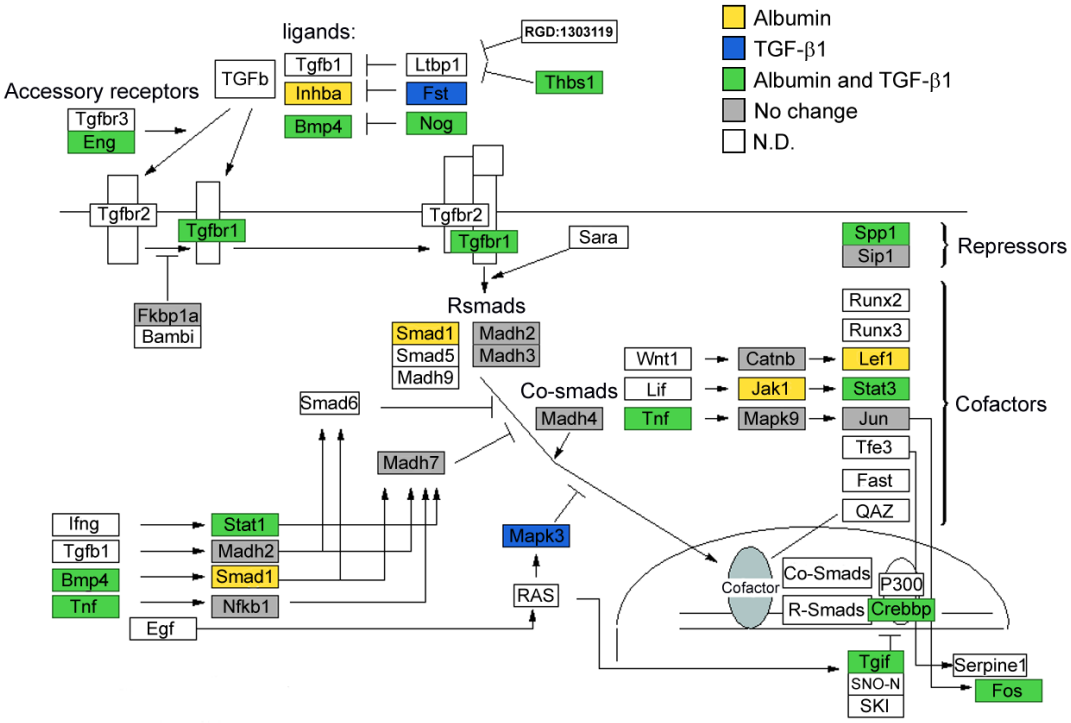
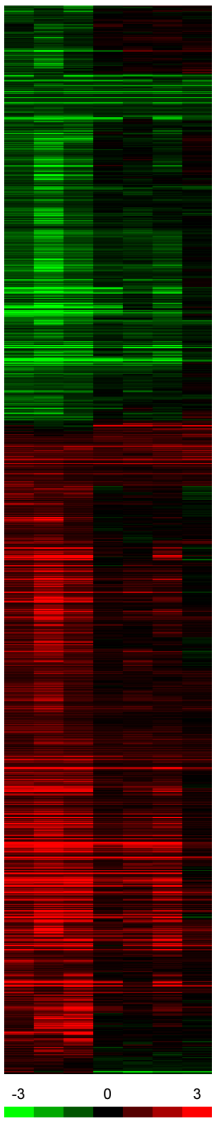


Figure 7. Albumin alters TGF-β pathway gene expression. TGF-β pathway map generated with GENMAPP software illustrating significant changes (>1.5 or <-1.5 fold change) in gene expression following albumin treatment in comparison to TGF-β1 treatment. Yellow labeled genes represent genes up or downregulated following albumin treatment, blue labeled genes represent genes up or downregulated following TGF-β1 treatment, and green labeled genes represent genes up or downregulated following both treatments. Gene pathway map created by Nurit Gal and Manny Ramirez, Copyright 2002, Gladstone Institute.

The above evidence indicates that TGF-β signaling is a key mediator of albumin-induced epileptogenesis. To determine if the global transcriptional response seen following albumin treatment is dependent on activation of the TGF-β signaling pathway, an additional set of microarray expression profiles were created using rats treated with albumin in the absence (n=3) or presence of TGF-βRI and II blockers (n=4, TGF-βR1 kinase activity inhibitor SB431542 and anti-TGF-βRII antibody) and sacrificed 24 hours following treatment. Although some changes in gene expression resulting from albumin treatment were still present following the blocker treatment, the majority of these changes were absent or attenuated following TGF-β pathway blocker treatment (Fig. 8A), confirming dependence of the albumin-induced transcriptional response on TGF-β signaling.

Gene ontology analysis was then used to reveal which biological processes were blocked following TGF-β pathway blocker treatment (Fig. 8A; for complete GO term annotation results see Supplementary Tables 5-8). Genes in the TGF-β signaling GO term demonstrated a dramatic suppression of the albumin-induced expression changes in the presence of TGF-β signaling blockers (Fig. 8B). In addition, TGF-β pathway blocker treatment prevented the albumin-induced modulation of genes involved in neuronal processes, immune response, and ion and cellular transport (Fig 8B). Several prominent signaling pathways including the NF-kappaB cascade, Jak-Stat cascade, and MAPKKK cascade were upregulated following albumin treatment, but did not show a similar upregulation following albumin treatment in the presence of TGF-β pathway blockers. Quantitative real-time PCR was also performed with these samples to confirm the microarray results (Fig. 8C). Indeed, TGF-β pathway blocker treatment completely blocked expression changes following albumin exposure for *Stat3* and *Glt-1* and partially blocked changes for *Cx43* and *GFAP*.

A.



GO Term	# genes	%	p-value
unattenuated response - downregulation			
transferase activity,transferring nitrogenous groups	3	3.2	5.3E-03
developmental process	16	17.0	4.1E-02
binding	41	43.6	1.3E-02
unattenuated response - upregulation			
translation regulator activity	9	5.6	7.8E-07
protein binding	62	38.5	4.7E-06
nucleotide binding	29	18.0	1.6E-04
intracellular signaling cascade	23	14.3	2.3E-04
isomerase activity	6	3.7	2.2E-03
cell motility	9	5.6	1.0E-02
nucleobase metabolic process	3	1.9	1.1E-02
intracellular transport	12	7.5	1.2E-02
cell death	12	7.5	2.1E-02
cytoskeleton organization & biogenesis	9	5.6	2.4E-02
cell proliferation	12	7.5	2.5E-02
transferase activity	20	12.4	3.6E-02
nucleic acid binding	27	16.8	4.8E-02
attenuated response - downregulation			
oxidoreductase activity	28	8.6	9.5E-06
vitamin binding	8	2.5	4.9E-04
nervous system development	23	7.0	6.8E-04
organic acid metabolic process	18	5.5	9.0E-04
ion transport	22	6.7	9.2E-04
electron transport	15	4.6	1.8E-03
cation binding	49	15.0	2.1E-03
transporter activity	29	8.9	2.4E-03
glutathione transferase activity	4	1.2	6.7E-03
extracellular matrix structural constituent	5	1.5	1.3E-02
cell growth	7	2.1	1.7E-02
cell adhesion	15	4.6	2.8E-02
transmission of nerve impulse	12	3.7	4.0E-02
cell proliferation	17	5.2	4.1E-02
phospholipase activity	4	1.2	4.3E-02
attenuated response - upregulation			
protein binding	201	41.1	5.2E-21
response to stress	72	14.7	2.4E-17
developmental process	126	25.8	1.5E-15
cell death	49	10.0	2.3E-11
cell motility	35	7.2	2.8E-11
cell proliferation	43	8.8	5.4E-08
cell adhesion	36	7.4	2.7E-07
cellular component organization & biogenesis	88	18.0	2.2E-06
intracellular signaling cascade	56	11.5	5.0E-06
immune system process	37	7.6	1.5E-05
chemotaxis	11	2.3	1.9E-04
cation homeostasis	15	3.1	3.2E-04
cell growth	12	2.5	8.1E-04
endocytosis	12	2.5	1.3E-03
cytokine production	9	1.8	1.7E-03
nucleotide binding	62	12.7	2.2E-03
nuclear transport	10	2.0	3.3E-03
cell cycle process	20	4.1	8.1E-03
carbohydrate binding	15	3.1	1.4E-02
response to hormone stimulus	10	2.0	1.4E-02
gene expression	73	14.9	1.8E-02
phospholipid binding	10	2.0	1.8E-02
enzyme linked receptor protein signaling pathway	13	2.7	2.0E-02
transferase activity, transferring glycosyl groups	11	2.3	2.2E-02
DNA-dependent DNA replication	6	1.2	2.3E-02
kinase activity	30	6.1	2.4E-02
extracellular matrix structural constituent	6	1.2	2.7E-02
phosphotransferase activity, alcohol group as acceptor	26	5.3	4.0E-02
cysteine-type endopeptidase activity	7	1.4	4.0E-02

B.

Gene symbol	fold change	
	albumin	album + blocke
TGF-β signaling		
Spp1	85.4	10.8
PAI-1	6.4	1.4
Stat3	6.0	1.3
Fos	4.7	1.0
Tgfb2	2.9	1.3
Tgif	2.7	1.6
Smad1	2.7	1.5
Jun	2.3	1.0
Bmp6	-2.5	-1.2
Immune response		
Ccl2	356.2	30.5
Ccl7	59.2	4.2
CD14	36.3	3.4
Tnfrsf12a	29.2	5.7
CD44	28.6	5.9
Gal	9.4	1.8
C5r1	5.2	1.2
Il1rap	4.9	1.0
Il1r2	2.1	1.1
Fn1	1.7	1.1
Ion and cellular transport		
Scn7a	13.9	2.4
Clic4	2.5	1.4
Kir6.1	1.9	1.2
SK2	-1.5	-1.2
Cx43	-1.6	-1.1
Chrm3	-2.0	1.0
Atp1a2	-3.0	1.0
Cx30	-5.4	-1.4
Cx26	-22.1	-2.0

C.

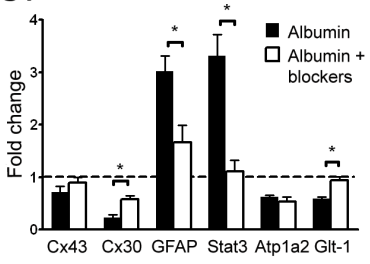


Figure 8. Blocking TGF-β signaling prevents albumin-induced gene expression.
A, Genomic expression analysis following treatment with albumin or albumin plus TGF-β receptor blockers. Significance analysis of microarrays (SAM) was performed with a false discovery rate (FDR) threshold of 9.2% and these genes are represented on the heat map. Gene ontology analysis was performed with DAVID for genes showing an attenuated [(albumin log2ratio) – (albumin + blocker log2ratio) > 0.5] or unattenuated response following treatment with albumin plus TGF-β receptor blockers in comparison to albumin treatment (see methods). **B,** Fold changes for specific genes from GO analysis. **C,** qPCR analysis for selected genes following albumin (n=3) or albumin plus TGF-β receptor blockers (n=4). Error bars indicate s.e.m and asterisks indicate $p<0.05$.

DISCUSSION

We have previously shown development of epileptiform activity after BBB opening or exposure to serum albumin (Seiffert, Dreier et al. 2004; Ivens, Kaufer et al. 2007). This study now extends these findings to include the appearance of spontaneous, prolonged and hypersynchronous interictal-like activity following treatment with albumin in a solution containing serum levels of electrolytes (aSERUM). Spontaneous activity was not observed when slices were treated with albumin or TGF- β in aCSF, probably due to the generally low excitability and lack of spontaneous activity of the deafferented slice preparation (Connors, Gutnick et al. 1982; Gutnick, Connors et al. 1982). Spontaneous activity has been shown to be rarely evoked in slices treated with albumin in aCSF (~10% of slices, see also (Seiffert, Dreier et al. 2004). The changes in electrolyte concentrations in the aSERUM solution (e.g. higher K+, lower Mg2+ and Ca2+) are probably sufficient to increase neuronal excitability such that the epileptiform activity, which characterizes the network upon activation, appears spontaneously. Importantly, spontaneous recurrent seizures followed by secondary generalization were also observed in some animals treated with albumin (Ivens, Kaufer et al. 2007). These observations combined with the appearance of epileptiform activity following albumin treatment *in vitro* likely reflect abnormal network epileptic network activity *in vivo*.

Since serum albumin is sufficient to induce epileptic-like activity, the initial hypothesis was that albumin enters the normally inaccessible CNS environment, binds to TGF-β receptors, and causes a cascade of events culminating in epileptiform activity. Thus study shows similar development of epileptiform activity following exposure to TGF-β1, demonstrating the importance of TGF-β pathway activation in this injury model. Furthermore, albumin binds to TGF-βRII and induces the phosphorylation of Smad2. Given the latent period prior to the appearance of epileptiform activity (ie epileptogenesis), it was hypothesized that BBB breakdown, albumin, and TGF-β1 share a common mechanism, specifically a transcriptional mechanism involving the TGF-β pathway. Indeed, clustering and gene ontology analysis revealed striking similarities across the three treatments. The most prominent finding from the microarray results is the identification of a key role for TGF-β signaling, with activation of TGF-β related genes seen in response to TGF-β1, albumin, and BBB breakdown. Blocking TGF-β signaling prevented the majority of the albumin-induced transcriptional responses, narrowing the gene list to identify the genes that are most relevant to epileptogenesis under these conditions. Furthermore, application of TGF-βR blockers suppressed the development of epileptiform neuronal activity following albumin or TGF-β1 treatment, highlighting this pathway as a novel therapeutic target.

Other significant findings from the microarray results include the early upregulation of genes involved in inflammatory processes and the delayed downregulation of genes involved in neuronal processes including synaptic transmission and ion transport. Features of CNS inflammation, such as glial and complement activation, cytokine production and adhesion protein expression, were all present in our array data. Upregulation of genes involved in activation of the NF-kappaB pathway and complement cascade reflects a significant innate immune response. In recent years, several studies have shed light on the importance of inflammatory processes in epilepsy. Both NF-kappaB pathway activation and complement activation have been reported in various epilepsy animal models (Rozovsky, Morgan et al. 1994; Rong and Baudry 1996; Gorter, van Vliet et al. 2006; Aronica, Boer et al. 2007; Lubin, Ren et al. 2007), as well as the involvement of other immune response genes including *Il6* (Balosso, Maroso et al. 2008), *Ccl2* (Calvo, Yoshimura et al. 1996; Manley, Bertrand et al. 2007), *Stat3* (Choi, Kim et al. 2003), and *Fn1* (Hoffman and Johnston 1998). A study by Rizzi et al., (2003), where status epilepticus (SE) was induced with kainic acid, found cytokines to be causally involved in the SE-induced neuronal damage, as cytokine synthesis preceded hippocampal neuronal injury and this injury only occurred when cytokines were produced (Rizzi, Perego et al. 2003).

Some immune-related genes have also been shown to play a role in neuronal functions. Cytokines such as interleukin-1β (*Il1b*) and tumor necrosis factor (*TNF*) have been shown to increase neuronal excitability. *Il1b* mediates increased neuronal glutamate release (Casamenti, Prosperi et al. 1999), induces phosphorylation of the NMDA NR2B subunit (Viviani, Bartesaghi et al. 2003; Balosso, Maroso et al. 2008) and prevents glutamate uptake by astrocytes (Hu, Sheng et al. 2000). *TNF* promotes recruitment of AMPA receptors lacking the Glur2 subunit to the neuronal membrane leading to increased calcium influx and also promotes endocytosis of GABA A receptors (Beattie, Stellwagen et al. 2002; Stellwagen, Beattie et al. 2005; Leonoudakis, Zhao et al. 2008). An upregulation of *TNF* was observed in the arrays from this study, indicating it may be involved in increasing neuronal excitability during epileptogenesis in addition to its immune related functions. Another immune related protein with a possible non-immune function following injury is C1q, the first component of the classical complement cascade. C1q has recently been shown to play a role in synapse elimination during development and in neurodegeneration (Stevens, Allen et al. 2007). Upregulation of several *C1q* subcomponents in the arrays may be associated with synaptic remodeling and neuronal loss following the generation of the epileptic focus (Tomkins, Friedman et al. 2007).

An imbalance of excitatory and inhibitory transmission resulting in increased network excitability is a feature of most epilepsy models. In this study, several GABA-A receptor subunits were downregulated while ionotropic glutamate receptor subunits were upregulated. Genes involved in glutamate transport (*Glt-1*) as well as voltage gated ion channels were also downregulated. These changes, appearing in the middle-late time points

predict an increase in network excitability. These results are also consistent with a previous microarray study examining gene expression changes during epileptogenesis following electrically induced SE (Gorter, van Vliet et al. 2006), which found downregulation of GABA-A and NMDA receptor subunits.

While many of the molecular changes revealed by our microarray study are shared with different epilepsy models, our study is the first to demonstrate that these changes, following direct vascular injury (i.e. BBB breakdown), are associated with brain exposure to serum albumin and are mediated via the TGF-β signaling pathway. Indeed, using TGF-β pathway blockers we were able to not only block the transcriptional response following albumin exposure *in vivo*, but also the development of epileptiform activity. Combined, these results present the TGF-β pathway as a novel therapeutic tool for preventing injury-related epileptogenesis.

Chapter 3 – Astrocytes are responsible for changes in potassium and glutamate buffering during albumin-induced epileptogenesis.

Introduction

An early study in 1964 by Kuffler and Potter first established the importance of astrocytes in regulating the extracellular environment of the brain and since then there has been accumulating evidence that glial cells influence neuronal function. Following neuronal activity, astrocytes play an important role in neurotransmitter recycling as well as buffering of extracellular potassium ions (Newman, Frambach et al. 1984; Oliet, Piet et al. 2001). Activity leads to a temporary increase in extracellular potassium, which is then cleared by astrocytic potassium channels. For glutamate and GABA, neurotransmitter recycling is achieved by astrocytic glutamate transporters as well as glutamine synthetase which converts glutamate into glutamine which can be transported back to neurons and converted to glutamate once again to be reused or converted into GABA (Bak, Schousboe et al. 2006).

Disruption of potassium buffering as well as the glutamate-glutamine cycle has been shown in neurological disorders such as epilepsy. Studies on epileptic tissue have found decreased expression of proteins involved in regulating extracellular potassium and glutamate (Hinterkeuser, Schroder et al. 2000; Schroder, Hinterkeuser et al. 2000) while increased levels of extracellular glutamate have been observed in epileptic foci (Glass and Dragunow 1995). One study has found a decrease in expression for the astrocytic transporters, Slc1a2 and Slc1a3, although other studies contradict these findings (Tessler, Danbolt et al. 1999; Proper, Hoogland et al. 2002). A more consistent finding has been the downregulation of glutamine synthetase which is responsible for converting glutamate into glutamine and has been proposed to be responsible for slowing of the glutamate-glutamine cycle and accumulation of glutamate in astrocytes and the extracellular space. The increase in extracellular potassium which would also lead to hyperexcitabilty, has been linked to downregulation of Kir potassium channels (Hinterkeuser, Schroder et al. 2000; Kivi, Lehmann et al. 2000; Schroder, Hinterkeuser et al. 2000).

In addition to these changes, there is also substantial reactive gliosis in epileptic tissue (Bordey and Sontheimer 1998). Reactive gliosis is prominent in response to a variety of pathologies and is characterized by hypertrophy of the astrocytic cell body and processes, which is associated with upregulation of the intermediate filament proteins GFAP and vimentin (Wilhelmsson, Li et al. 2004; Pekny and Nilsson 2005). Following albumin treatment, we have also observed upregulation of both of these genes and their products (Fig. 6 and (Ivens, Kaufer et al. 2007) along with many other changes in astrocytic genes. We therefore decided to investigate the role of astocytes in albumin-induced epileptogenesis and this chapter will focus on albumin-induced changes in glutamate and potassium buffering.

Results

Microarray data from DOC and albumin treated animals (n = 3 from each treatment) was analyzed in order to evaluate changes in cell-type specific genes during the first 48 hours following treatment, which constitutes the epileptogenesis time window (Seiffert, Dreier et al. 2004; Ivens, Kaufer et al. 2007). Given that the initial functional annotation analysis (Fig. 6F) revealed many changes in astrocytic genes, we wanted to compare gene expression in neurons and astrocytes.

A recent article by Cahoy et al. described a cell type specific database of gene expression for neurons, astrocytes and oligodendrocytes. This database was used to classify the microarray gene expression changes into astrocytic and neuronal categories. A comparison between these two categories revealed a greater number of genes in the astrocytic category (Fig. 9A). Given that astrocytes are important for glutamate, ion and water homeostasis, genes associated with these processes were then analyzed. The heat map in figure 9B shows individual gene expression changes across time. These genes include glutamate transporters, glutamine synthetase, potassium channels, and connexins.

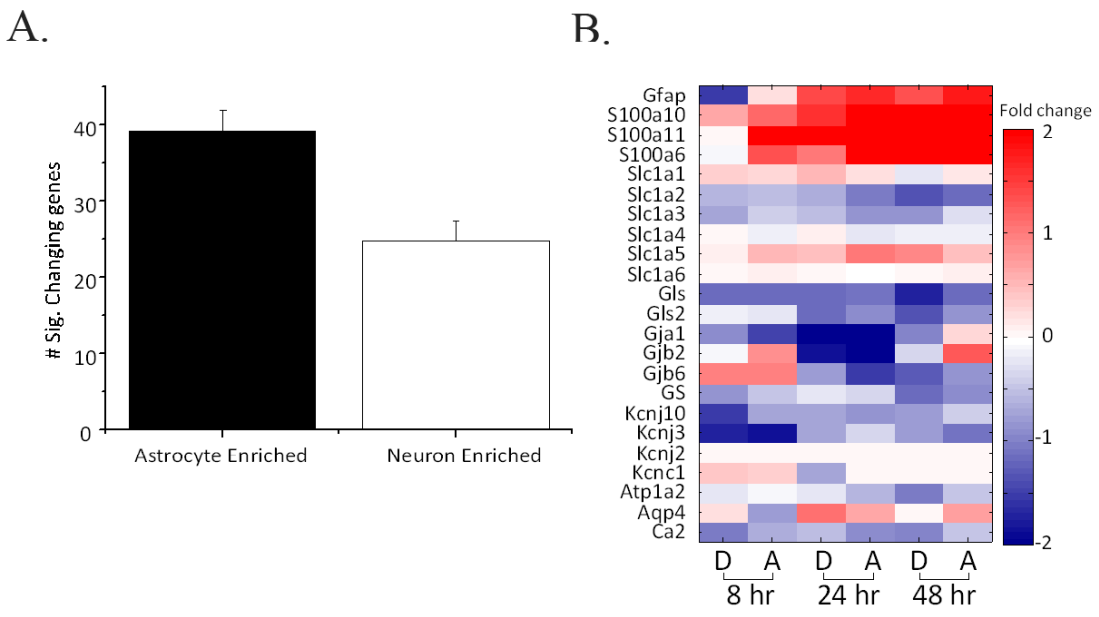


Figure 9. Transcriptional changes in astrocytes following albumin and BBB disruption. **A,** Average number of gene transcripts up- or down-regulated by more than 150% grouped by cell type across all time points. **B**, Sham-normalized expression levels of mRNA for genes preferentially expressed in astrocytes at 8, 24, and 48 hours following treatment with albumin and BBB disruption with DOC (D = DOC and A= albumin).

While Kcnj10 (Kir4.1), an inward rectifying potassium channel predominantly expressed in astrocytes was downregulated, other neuronal potassium channels such as Kcnj2 and Kcnc1 (Butt and Kalsi 2006) were not altered following DOC or albumin treatment. Members of the solute carrier family 1, subfamily A, Slc1A2 (Glt-1) and Slc1A3 (Glast), were also downregulated. These genes encode glutamate transporters expressed by astrocytes (Chaudhry, Lehre et al. 1995; Su, Leszczyniecka et al. 2003). On the other hand, another glutamate transporter expressed by neurons, Slc1A1 (Rothstein, Martin et al. 1994), showed no change in expression. In addition to these glutamate transporters, other astrocytic genes involved in glutamate homeostasis were downregulated as well. These include glutaminase (Gls), glutaminase 2 (Gls2), and glutamine synthetase (GS) (Derouiche and Frotscher 1991). Finally, there was also a substantial downregulation of the astrocyte specific connexin genes Gja1, Gjb2 and Gjb6 which correspond to connexin 43, connexin 26, and connexin 30, respectively. Combined, these results indicate decreased potassium buffering, glutamate transport and spatial buffering, all of which contribute to hyperexcitability.

In order to confirm that these changes in gene expression were confined to astrocytes, primary cortical cultures enriched for either astrocytes or neurons were established. The purity of these cultures was validated by immunostaining using the cell specific markers GFAP (a marker for astrocytes) and NeuN (a marker for neurons). Figure 10 shows that cells from cultures enriched for astrocytes are mostly GFAP positive (Fig 10A) and NeuN negative (Fig. 10B), while cells from cultures enriched for neurons are mostly NeuN positive (Fig. 10C) and GFAP negative (Fig. 10D).

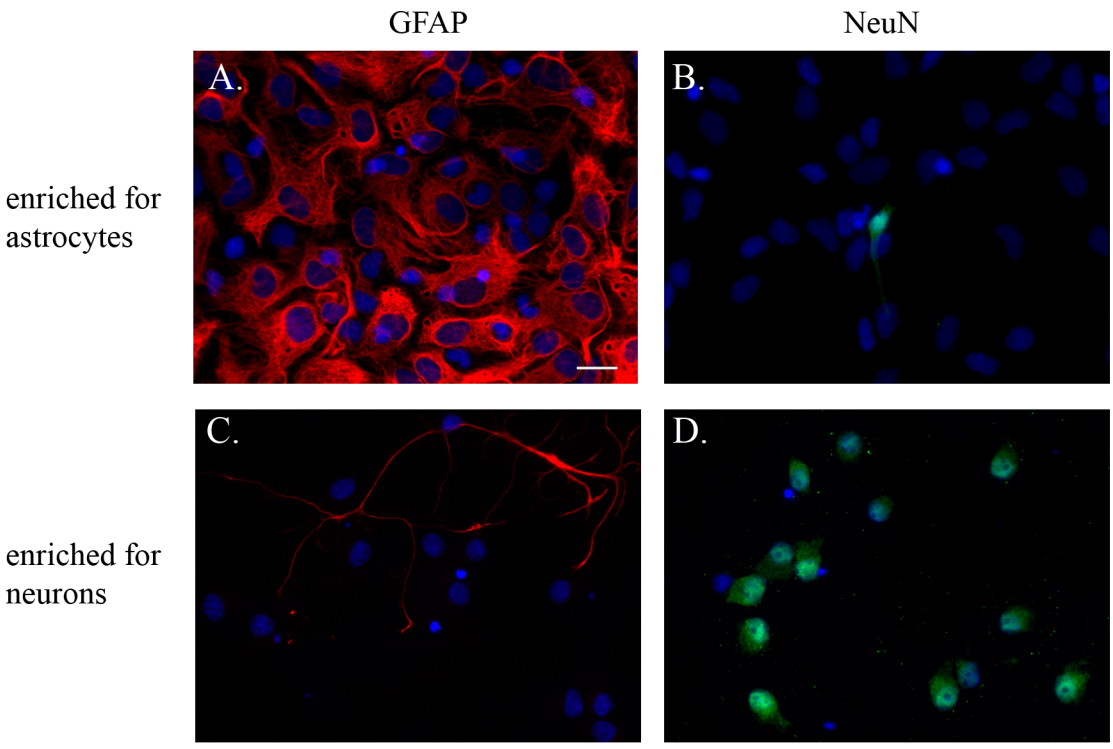


Figure 10. Primary astrocytic and neuronal cultures. Astrocyte enriched cultures immunolabeled for GFAP (A, red), NeuN (B, green) and DAPI (A and B, blue). Neuron enriched cultures immunolabeled for GFAP (C, red), NeuN (D, green) and DAPI (C and D, blue). Scale bar corresponds to 20 µm.

These cultures were treated with 0.4 mM albumin for 24 hours and changes in gene expression were then evaluated for each cell type. While most of the genes selected showed significant downregulation in the astrocytic cultures, none of these genes showed any change in the neuronal cultures in response to albumin treatment (Fig. 11). The three astrocyte specific connexins, Cx43, Cx26 and Cx30, showed the greatest downregulation with more than a two fold change in expression. The glutamate transporter, Glast (Slc1A3) as well as glutamine synthetase (GS) were also downregulated specifically in astrocytes while there was no change in expression for the glutamate transporter Slc1A2. This data confirms the results from the microarrays demonstrating astrocyte specific changes in gene expression during albumin-induced epileptogenesis.

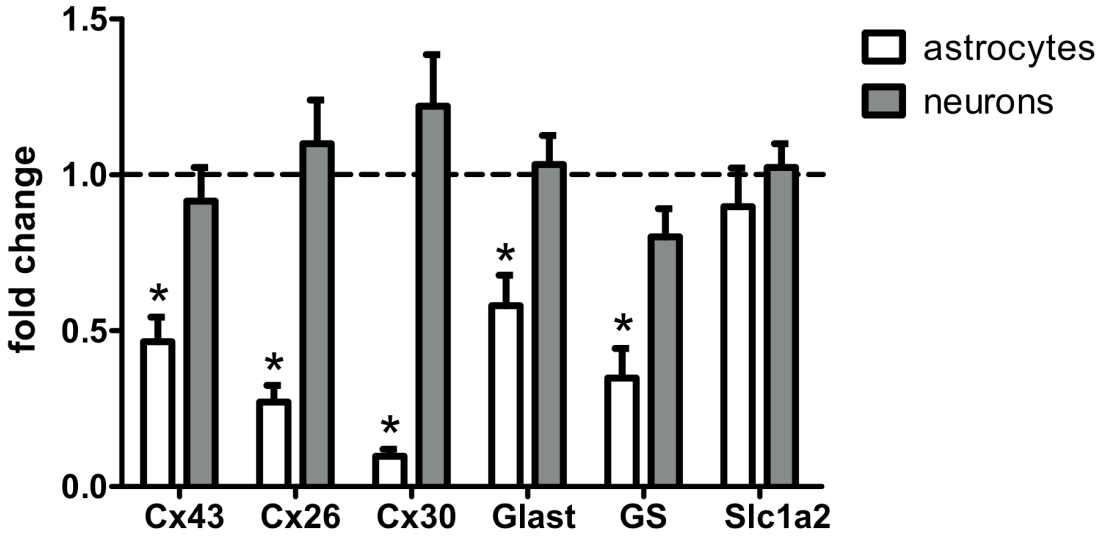


Figure 11. Changes in astrocytic genes regulating potassium and glutamate homeostasis. qPCR analysis for select genes. Astrocyte and neuron enriched primary cultures were treated with 0.4 mM albumin for 24 hr. Fold changes are relative to untreated (neurons) or serum deprived (astrocytes) controls.

Discussion

Altered astrocytic responses in epilepsy are well documented (Tian, Azmi et al. 2005; Wetherington, Serrano et al. 2008)., but our experiments have shown for the first time that an astrocytic reaction (manifested as enhanced transcription and expression of the glial fibrillary acidic protein (GFAP) was observed during the first day following albumin exposure and preceded the appearance of epileptic activity (Ivens, Kaufer et al. 2007). The possible involvement of albumin in astrocytic activation and proliferation is also supported by previous studies showing serum albumin inducing the proliferation of fibroblasts (Tigyi, Dyer et al. 1994) as well as calcium signaling and DNA synthesis in cultured astrocytes (Nadal, Fuentes et al. 1995).

Glial cells are responsible for homeostasis of the extracellular environment (i.e. uptake of glutamate and potassium) under normal conditions and for the initiation of inflammatory processes in the central nervous system when activated following injury. Importantly, the early astrocytic response, before epileptic activity is observed, further strengthens the putative role of these cells in the epileptogenic process itself. The microarray data shown here demonstrates downregulation of the astrocytic glutamate transporters, Slc1a2 and Slc1a3, glutamine synthetase, the inward rectifier potassium channel Kcnj10 and connexins Cx26, Cx30 and Cx43. Furthermore, most of these results were also observed in primary astrocytic cultures treated with albumin.

The changes in gene expression shown here are consistent with other epilepsy models and point to a decrease in glutamine uptake and recycling as well as spatial buffering. Overall, the largest change in gene expression *in vitro* was the downregulation of the astrocytic connexin genes. In recent years, there has been increased interest in the role of gap junctions in epilepsy. Changes in gene expression for various connexins have been reported but the findings are contradictory. Studies on astrocytic gap junctions have centered on connexin 43. In human epileptic tissue, both an increase (Naus, Bechberger et al. 1991; Fonseca, Green et al. 2002; Collignon, Wetjen et al. 2006) as well as no change (Elisevich, Rempel et al. 1997) in Cx43 protein and/or gene expression has been reported. In animal models, on the other hand, Cx43 expression decreased or no change was reported(Khurgel and Ivy 1996; Elisevich, Rempel et al. 1997; Sohl, Guldenagel et al. 2000; Li, Shen et al. 2001; Xu, Zeng et al. 2009). Similarly for connexin 30, there was either an increase (Condorelli, Mudo et al. 2002) or no change (Sohl, Guldenagel et al. 2000)in gene expression reported.

This data is further confounded by studies of gap junction blockers which appear to be anticonvulsant (Jahromi, Wentlandt et al. 2002; Nemani and Binder 2005; Medina-Ceja, Cordero-Romero et al. 2008). These studies are difficult to interpret, however, as

specificity of these blockers has not been established. It may actually be beneficial to block neuronal gap junctions in order to prevent hypersynchronous activity whereas blocking astrocytic gap junctions will most likely promote hypersynchronous activity through decreased spatial buffering. In order to elucidate these effects, knockout mice have been generated to investigate individual connexins. A recent study by Wallraff et al. used mice deficient in connexins 30 and 43 to demonstrate a role for astrocytic gap junctions in potassium buffering. This group measured potassium buffering in these animals with potassium-selective microelectrodes and showed a partial deficit in potassium clearance in the absence of astrocytic gap junctions. Furthermore, they also showed that elimination of these gap junctions lowered the threshold for development of epileptiform activity, which is in agreement with the results presented here.

While our gene expression data supports a decrease in glutamate and potassium buffering, it is also important to confirm that these changes are associated with altered cellular function. In follow up experiments, our group investigated extracellular glutamate and potassium clearance in cortical slices following a 24 hour albumin treatment *in vivo* (David, Cacheaux et al. 2009). Electrophysiological recordings confirmed the reduction in both extracellular potassium and glutamate following neuronal activity in albumin treated animals. A NEURON model was then used to predict if either or both of these deficits would have an effect on excitatory postsynaptic potentials (EPSPs). The model predicted that the reduction in potassium buffering is the major component underlying neuronal excitability induced by albumin treatment.

We did observe a downregulation of the inward rectifying potassium channel Kcnj10 *in vivo* which may play a major role in reduced potassium buffering. The lack of specific blockers which target these channels have made it challenging to test these effects. However, knockout mice lacking Kcnj10 channels display development of seizure activity early on, indicating an important role for this channel in epileptogenesis (Djukic, Casper et al. 2007). These results as well as our own have added to the growing literature demonstrating the importance of astrocytic buffering in modulating neuronal function.

Chapter 4 – Albumin induces activation of different TGF-β signaling pathways in neurons and astrocytes.

Introduction

The TGF-β family is comprised of pleiotropic cytokines which are important for cell growth, embryogenesis, differentiation, morphogenesis, wound healing, immune response and apoptosis in many different cell types (Gold and Parekh 1999; Blobe, Schiemann et al. 2000). Canonical TGF-β signaling is mediated by the serine threonine kinase receptors TGF-βR1 and TGF-βR2. Pathway activation begins once TGF-β1 binds to TGF-βR2. This receptor then phosphorylates TGF-βR1, which subsequently phosphorylates Smad2/3. Smad2/3 is then able to form a complex with Smad4 and translocate into the nucleus and regulate transcription (Massague and Gomis 2006).

While TGF-β1 is expressed at low levels in the normal brain, it is significantly upregulated following injury (Lindholm, Castren et al. 1992). TGF-β1 is thought to be neuroprotective (Prehn, Peruche et al. 1993; Ruocco, Nicole et al. 1999; Brionne, Tesseur et al. 2003) as it upregulates the transcription factor NFκB which then upregulates genes encoding the anti-apoptotic proteins BCL-2 and BCL-XL (Kim, Kim et al. 1998; Zhu, Yang et al. 2002). Recently, a study by Konig et al. demonstrated that in neurons, TGF-ß1 is able to activate the canonical Alk5/Smad2/3 pathway as well an alternative pathway mediated by the TGF-β type I receptor Alk1 which phosphorylates Smad1/5/8 (Fig. 12).

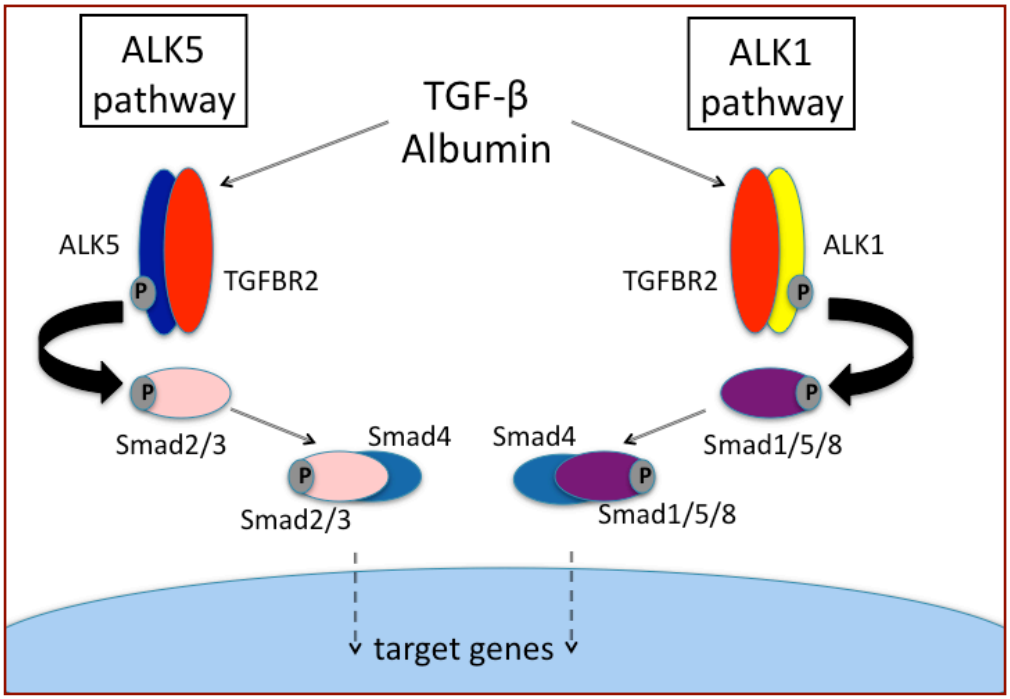


Figure 12. Schematic of TGF-β signaling pathways.

Alk1 is expressed mainly in the developing vascular system and plays an important role in endothelial cell development (David, Mallet et al. 2007). The Konig study showed for the first time that neurons and astrocytes also express this receptor. Using neuronal and astrocytic cultures they showed that while Alk5 is expressed at higher levels in astrocytes, Alk1 expression is higher in neurons. In addition, this group observed an increase in Alk1 expression and downstream signaling following excitotoxic injury in neurons, which led to NFκB activation. This data indicates that while both pathways are present in neurons, the Alk1 pathway is responsible for neuroprotection following injury.

In chapter 2, we demonstrated that albumin binds to TGF-βR2 and induces Smad2 phosphorylation. We have also shown that glial activation and induction of astrocytic genes precedes changes in neuronal genes during albumin-induced epileptogenesis even though TGF-β receptors are found on both cell types. Differential pathway activation by albumin in different cell types could account for these observations. Therefore, we hypothesized that like TGF-β1, albumin primarily activates the canonical Alk5 pathway in astrocytes and the alternate Alk1 pathway in neurons.

Results

TGF-β1 induces cell specific Smad phosphorylation

TGF-β1-induced signaling involves phosphorylation of Smad proteins by TGF-β type I receptors. Primary cortical cultures enriched for astrocytes or neurons were treated with 10 ng/mL TGF-β1 to verify phosphorylation in this system. While TGF-β1 treatment in astrocytes resulted in a small increase in Smad1 phosphorylation after 4 hours, the same treatment resulted in a much larger increase in Smad2 phosphorylation (Fig. 13). When primary neurons were treated with TGF-β1 for similar periods of time, the inverse was observed. There was an increase in phosphorylation of both Smad proteins but there was a much greater increase in phosphorylation of Smad1 than Smad2. These results confirm previous findings (Konig, Kogel et al. 2005) that there is preferential phosphorylation of Smad1 in neurons and Smad2 in astrocytes.

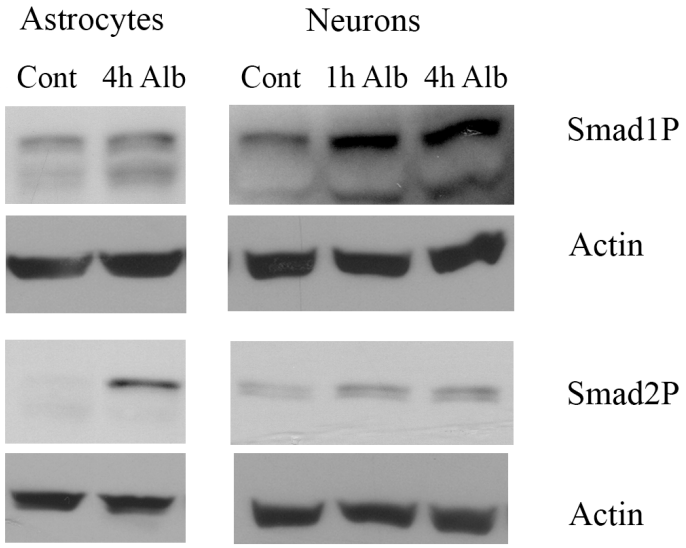


Figure 13. TGF-β1 treatment induces Smad1 and Smad2 phosphorylation in astrocytes and neurons. Western blots for Smad1P and Smad2P in neurons and astrocytes treated with 10 ng/mL TGF-β1 for 1-4 hr. Actin is used as a loading control. Experiments were run in triplicate.

Albumin elicits cell specific changes in TGF-ß receptors

To characterize albumin induced TGF-β signaling in different cell types, changes in TGF-β receptor expression were first evaluated. It was hypothesized that albumin would elicit activation of the canonical TGF-β pathway in astrocytes consisting of Alk5 mediated signaling while the alternative pathway mediated by Alk1 would be activated in neurons. When primary astrocytes were treated with 0.4 mM albumin for 24 hours there was an increase in Alk5 expression but no change in Alk1 expression (Fig. 14). Following the

same treatment in primary neurons, there was no change in Alk5 expression and a small increase in Alk1 expression. It is interesting to note that control levels of Alk5 are greater in neurons than in astrocytes, possibly indicating greater basal activation of the canonical pathway in neurons. However, this could also be a result of serum deprivation in the astrocytes.

The common component of both TGF-β pathways is TGF-βR2. When primary cultures were exposed to albumin, there was a decrease in TGF-βR2 for both neurons and astrocytes (Fig. 14). In addition to the expected band at 70 kDa, an additional band appeared following albumin treatment. This band has a lower molecular weight and was more prominent in the neuronal cultures. Given that a polyclonal antibody was initially used, the specificity of this extra band was verified with a monoclonal antibody which produced the same result. Possible explanations for this band include protein degradation, posttranslational modifications and alternative splice variants.

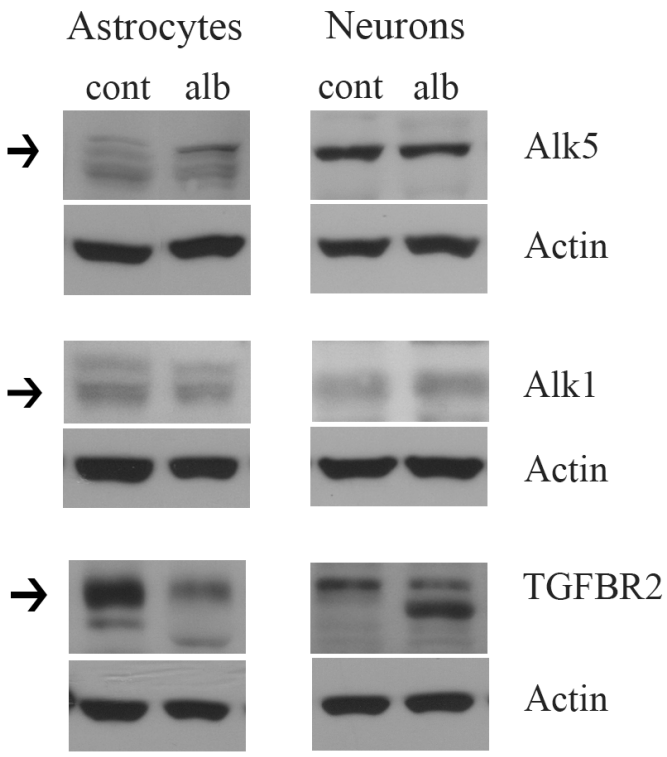


Figure 14. TGF-β receptor expression changes in astrocytes and neurons. Western blots for Alk5 (TGF-β R1), Alk1, and TGF-βR2 in neurons and astrocytes treated with 0.4 mM albumin 24 hr. Arrows indicate the position of the expected band on the blot. Actin is used as a loading control. Experiments were run in triplicate.

Albumin elicits cell specific changes in Smad phosphorylation

Changes in TGF-β receptors should also lead to changes in downstream phosphorylation of Smad proteins. Levels of Smad 1 and 2 phosphorylation were investigated in primary astrocytic and neuronal cultures treated with 0.4 mM albumin. When these cells were treated with albumin for a short period of time (4 hours) there was an increase in Smad2 phosphorylation in the astrocytes and an increase in Smad1 phosphorylation in the neurons, as predicted (Fig. 15).

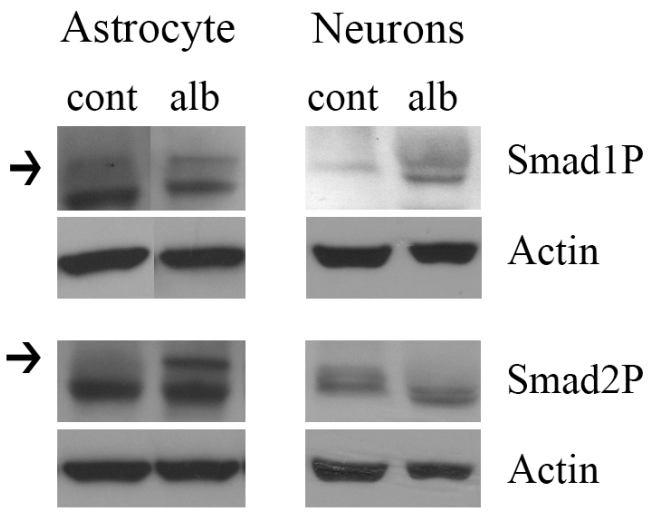


Figure 15. Differential Smad1 and 2 phosphorylation in astrocytes and neurons following a short exposure to albumin. Western blots for Smad1P and Smad2P in neurons and astrocytes treated with 0.4 mM albumin for 4 hr. Arrows indicate the position of the expected band on the blot. Actin is used as a loading control. Experiments were run in triplicate.

When these cells were treated with albumin for a longer period of time (24 hours) there was still an increase in Smad1 phosphorylation but no change in Smad2 phosphorylation in the neurons (Fig. 16). On the other hand, Smad2 phosphorylation went back to control levels in the astrocytes, but there was now a substantial increase in Smad1 phosphorylation.

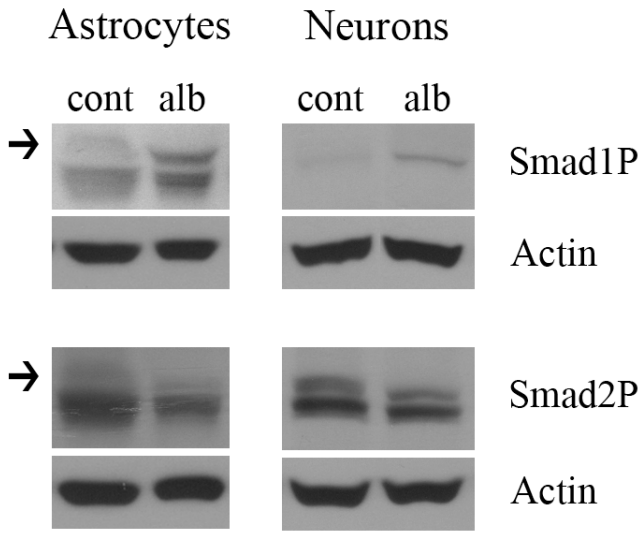


Figure 16. Increased Smad1 phosphorylation in astrocytes and neurons 24 hr following albumin treatment. Western blots for Smad1P and Smad2P in neurons and astrocytes treated with 0.4 mM albumin. Arrows indicate the position of the expected band on the blot. Actin is used as a loading control. Experiments were run in triplicate.

TGF-β pathway blockers prevent albumin-induced phosphorylation

Given that albumin has cell specific effects on TGF-β receptor expression and Smad phosphorylation, the next step was to prevent these changes using TGF-β pathway blockers. The blockers used were SB431542 and Losartan. SB431542 is a TGF-β type I receptor kinase inhibitor which should block Alk5 signaling while Losartan is an angiotensin type II receptor antagonist which has been shown to block TGF-β signaling (Campistol, Inigo et al. 1999; Khalil, Tullus et al. 2000). The effects of these blockers were evaluated in primary astrocytes as the working hypothesis is that astrocytes are the first cells to be affected during albumin-induced epileptogenesis. Primary astrocytes were first incubated with either 10 μM Losartan or 30 μM SB431542 for an hour before addition of either albumin or TGF-β1. As previously shown for a 4 hour treatment (Fig. 15), there was an increase in Smad2 phosphorylation following albumin treatment (Fig. 17). Treatment with Losartan plus albumin, however, partially blocked the increase in Smad 2 phosphorylation while treatment with SB431542 completely prevented this increase. As a positive control astrocytes were also treated with TGF-β1 in the absence and presence of SB431542. As expected the TGF-β1 induced increase in Smad2 phosphorylation was completely blocked by the addition of SB431542.

In addition to an increase in Smad2 phosphorylation, albumin also induced an increase in Smad1 phosphorylation 24 hours following albumin treatment (Fig. 16). However, this increase was not prevented by treatment with either SB431542 or Losartan.

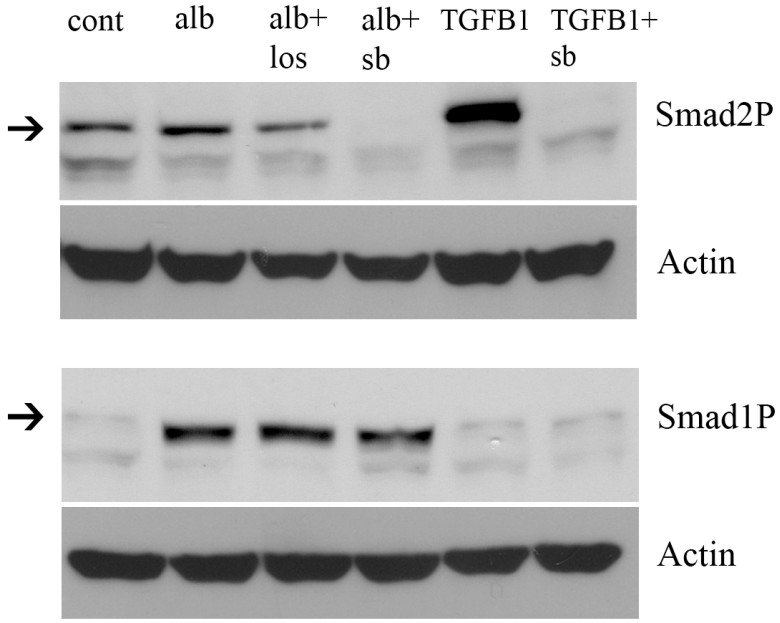


Figure 17. TGF-β pathway blockers prevent Smad2 phosphorylation in astrocytes. Western blots for Smad2P and Smad1P in astrocytes treated with 0.4 mM albumin for 4 hr and 24 hr, respectively in the presence and absence of TGF-β pathway blockers. Losartan (10 um) and SB431542 (30 um) were applied 1 hr before albumin treatment. TGF-β1 treatment (10ng/mL) was used as a positive control. Arrows indicate the position of the expected band on the blot. Actin is used as a loading control. Experiments were run in triplicate.

Discussion

In support of the initial hypothesis, this study demonstrates that albumin does indeed activate the canonical Alk5-Smad2/3 pathway in astrocytes while activating the alternate Alk1-Smad1/5/8 pathway in neurons. The results are summarized in figure 18. After a 4 hour treatment with albumin, there is an increase in Smad2 phosphorylation in astrocytes and an increase in Smad1 phosphorylation in neurons while at the 24 hour time point both cell types show an increase in only Smad1 phosphorylation. Changes in receptor expression also vary with an increase in Alk5 in astrocytes, a small increase in Alk1 in neurons and a decrease in TGF-βR2 in both cell types. Finally, treatment with the TGF-β pathway blockers attenuated the astrocytic increase in Smad2 phosphorylation.

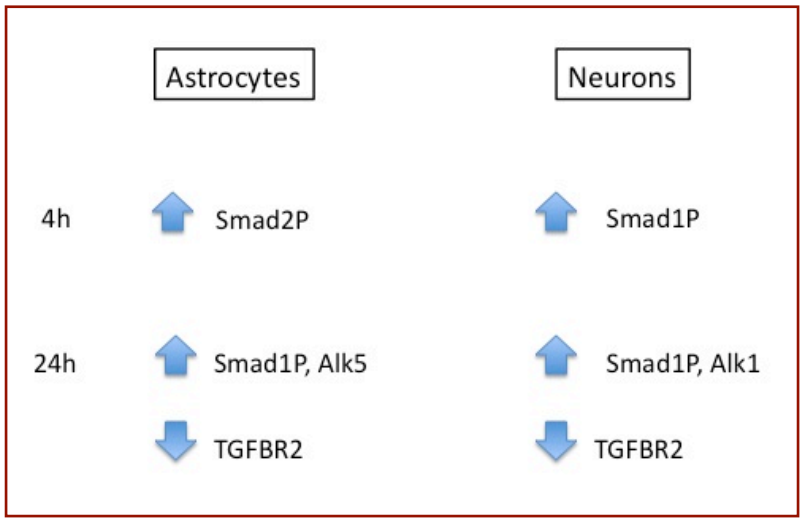


Figure 18. Summary of cell-type specific changes in TGF-β signaling.

Interestingly, TGF-β1 is generally considered to have a protective function. Previous studies have demonstrated the ability of TGF-β1 to prevent glutamate neurotoxicity *in vitro* and protect against ischemic injury *in vivo* (Prehn, Backhauss et al. 1993; Henrich-Noack, Prehn et al. 1996; Ruocco, Nicole et al. 1999). On the other hand, studies have also suggested TGF-β1 may not always be neuroprotective. Transgenic mice overexpressing TGF-β1 specifically in astrocytes (Wyss-Coray, Feng et al. 1995) developed seizures (along with overproduction of extracellular matrix components, severe communicating hydrocephalus, motor incoordination, and early runting). Furthermore, a recent study has demonstrated that blockade of TGF-β-Smad2/3 signaling in peripheral macrophages in a mouse model for Alzheimer's disease results in marked attenuation of cerebrovascular-amyloid deposits (Town, Laouar et al. 2008). These studies along with our work illustrate

the complexity and importance of TGF-β signaling in neurological diseases. Such complexity may be due to the presence of different combinations of TGF-β receptors resulting in activation of different downstream targets.

One of the most surprising results in this study was the appearance of an extra band in the TGF-βR2 western blot following albumin treatment which was more pronounced in the neurons. This band could correspond to protein degradation, other posttranslational modifications, or a different splice variant. There have been reports of an alternative splice variant, TGFβR2-B, which has an insertion of 25 amino acids in the ligand-binding domain (Suzuki, Shioda et al. 1994; Hirai and Fijita 1996; Krishnaveni, Hansen et al. 2006). While TGF-βR2 is normally only responsive to TGF-β1, TGFβR2-B is responsive to TGF-β1 and TGF-β2 (Rotzer, Roth et al. 2001). Even though this isoform does not correspond to the extra band observed here, it illustrates a role for alternative splicing of the TGF-βR2 gene. Another example of changes in this gene comes from the cancer literature where gastric cancer cell lines express a truncated form of TGF-βR2 which is unresponsive to TGF-β1 (Yang, Kang et al. 1999). Since albumin downregulates TGF-βR2 which presumably is the result of negative feedback, this extra band could represent a truncated receptor which would be another way to terminate the signal.

In addition to changes in TGF-βR2, another unexpected result was the transient increase in Smad2 phosphrylation and the later increase in Smad1 phosphorylation in astrocytes. Whereas there was an increase in Smad2 phosphorylation *in vivo* (Fig. 4) 24 hours following albumin treatment, there was no change *in vitro* at the same time point. This could be due to the fact that astrocytes in vitro need to be cultured in serum which is different from astrocytes *in vivo*, or a niche-dependent response *in vivo*. In addition, the time points are difficult to compare as one would expect a faster response in cells than in tissue where albumin needs to more time to diffuse into cells.

The increase in Smad1 phosphorylation, on the other hand, is probably involved in astrogliosis. Two studies have found that in neural stem cells, an increase in Stat3 leads to transcription of Bmp2 which then induces activation of Smad1. Activated Smad1 is able to form a complex with the co-activator p300 and Stat3 to induce transcription of GFAP which leads to the generation of astrocytes (Nakashima, Yanagisawa et al. 1999; Fukuda, Abematsu et al. 2007). The microarray data presented in Chapter 2 show a large upregulation in Stat3 (Fig. 8) 24 hours following albumin treatment as well as upregulation of Bmp2 (data not shown). These changes in combination with the astrocytic increase in Smad1 phosphorylation probably account for at least a portion of the astrogliosis observed during albumin-induced epileptogenesis.

Since TGF-β1 induction of the Alk1 pathway has been shown to be neuroprotective, this might also be the case for albumin-induced Alk1 signaling. A great deal of evidence points to an astrocytic response as the precipitating event in albumin-induced epileptogenesis. It would therefore be beneficial to target the Alk5 pathway in order to block epileptogenesis. I have already shown that blocking Alk5 kinase activity plus TGF-βR2 prevents the development of epileptiform activity and in this chapter I have demonstrated that blocking Alk5 kinase activity by itself preventss Smad2 phosphorylation in astrocytes. In addition, Losartan also partially blocked Smad2 phosphorylation. Losartan is an angiotensin-II type 1 receptor antagonist which is already in clinical use as a treatment for hypertension. It has been shown to decrease levels of TGF-β1 (Campistol, Inigo et al. 1999; Khalil, Tullus et al. 2000) and therefore might be a good candidate as a possible treatment for injury-induced epilepsy.

Conclusion

BBB breakdown is a hallmark of vascular injury in the brain and is observed in numerous neurological diseases including traumatic brain injury, stroke and neurodegenerative diseases (Neuwelt 2004; Abbott, Ronnback et al. 2006; Oby and Janigro 2006; Zlokovic 2008). Compromise of the BBB is triggered by preceding processes that in turn cause vascular injury. For example, perivascular astrocytes or perivascular microglia could be activated by an initial precipitating event (trauma or ischemia) and cause vascular injury leading to serum albumin extravasation into the brain parenchyma. We have previously shown development of cortical dysfunction – specifically, hypersynchronous neuronal activity following BBB opening or exposure to serum albumin (Seiffert, Dreier et al. 2004; Ivens, Kaufer et al. 2007). Cortical dysfunction was followed by reduced dendritic branching and neuronal loss several weeks following either treatment (Tomkins, Friedman et al. 2007).

In this thesis, I first demonstrate that albumin-induced epileptiform activity is mediated by activation of TGF-β receptors. Albumin binds to TGF-ßR2 and activates downstream signaling resulting in a transcriptional response. Disruption of the BBB, treatment with albumin and treatment with TGF-β1 all result in similar transcriptional profiles indicating a common pathway. These transcriptional profiles revealed changes in many different genes related to excitatory and inhibitory neurotransmission, inflammation and astrocytic cell function. I then investigated the role of astrocytic activation and found a downregulation in genes related to glutamate and potassium buffering in astrocytic but not in neuronal enriched primary cultures predicting disruption of both glutamate and potassium buffering. Finally, I examined activation of different TGF-β pathways in astrocytes and neuorns, and found cell specific changes in response to albumin. While albumin primarily activates the canonical TGF-β pathway mediated by Alk5 in astrocytes, albumin primarily activates an alternate TGF-β pathway mediated by Alk1 in neurons. Figure 18 presents a summary of the data presented here.

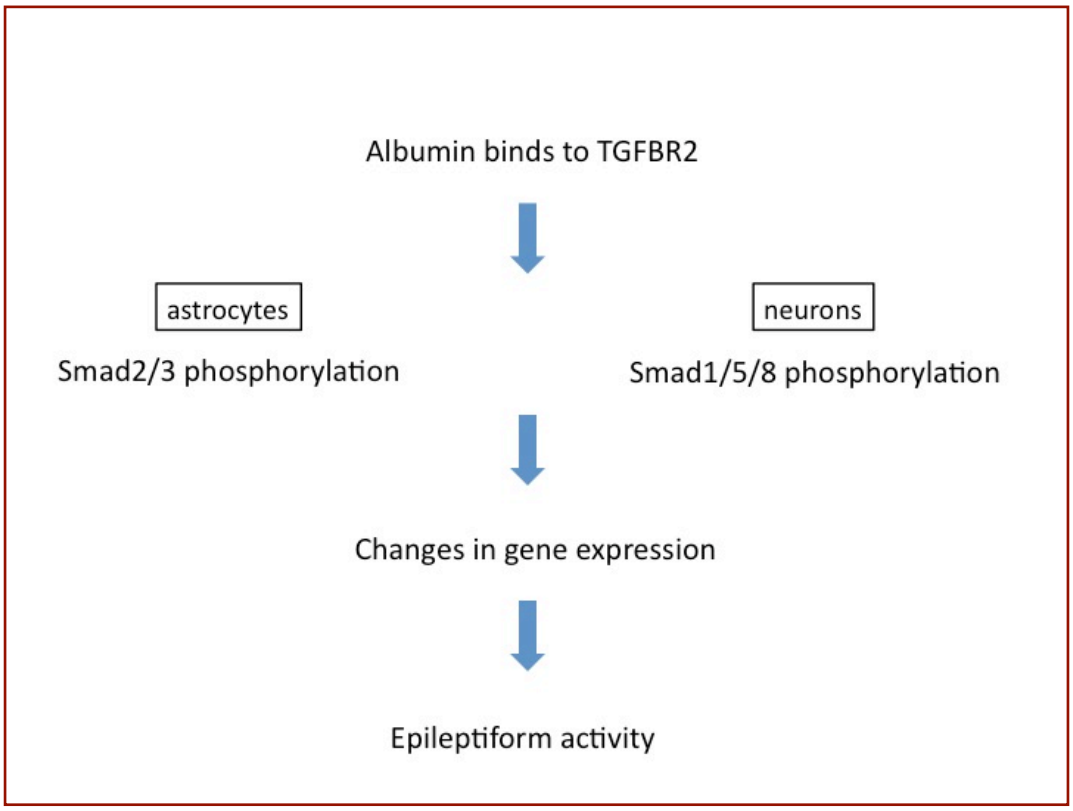


Figure 18. Proposed model for albumin-induced epileptogenesis.

While there are many antiepileptic drugs available, approximately one third of epilepsy patients do not respond to any drug (Sander 2003). This work as a whole has identified a novel pathway important for the development of injury-induced epileptogenesis, providing a viable therapeutic target. Additional work will be needed to determine whether TGF-β signaling is also important in other epilepsy models. TGF-β1 is produced in response to most types of injury and therefore it is plausible that this pathway could also serve as a therapeutic target for other types of epilepsy. Furthermore, identification of cell type specific pathways will aid in the development of more targeted antiepileptic drugs.

MATERIALS AND METHODS

***In vivo* preparation:** All experimental procedures were approved by the animal care and use ethical committees at Charité University Medicine, Berlin and Ben-Gurion University of the Negev, Beer-Sheva. The *in vivo* experiments were performed as previously described (Seiffert, Dreier et al. 2004; Ivens, Kaufer et al. 2007). In short, adult male Wistar rats (120-250 gr) were anesthetized and placed in a stereotactic cage, a 4 mm diameter bone window was drilled over the somatosensory cortex, and the dura was opened. The underlying cortex was then perfused with artificial cerebrospinal fluid (aCSF, composition in mM: 129 NaCl, 21 NaHCO3, 1.25 NaH2PO4, 1.8 MgSO4, 1.6 CaCl2, 3 KCl, 10 glucose) supplemented with either deoxycholic acid (DOC, 2 mM, Sigma-Aldrich, Steinheim, Germany), bovine serum albumin (BSA, 0.1-0.2 mM, Merck, Darmstadt, Germany) corresponding to 25% of serum albumin concentration, or with TGF-β1 (10 ng/ml, Peprotech, Rocky Hill, NJ) for 30 min. Sham-operated animals (perfused with aCSF) served as controls. Only rats with no apparent injury to the cortical surface or bleeding from cortical vessels (as seen under the surgical microscope) at the end of the procedure were used.

In order to investigate transcriptional changes occurring during the epileptogenesis time window (before the development of epileptiform activity), animals were sacrificed 7/8, 24, or 48 hours following treatment. RNA isolated from these animals was used for microarray and qRT-PCR analyses described below. As robust changes in gene expression were observed after the 24 hour treatments, a second set of animals including sham-operated controls and animals treated with BSA (0.1 mM) or BSA plus TGF-βR blockers (TGF-βRII antibody, 50 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA; SB431542, 100 μM, TGF-βRI kinase activity inhibitor, Tocris, Bristol, UK) were sacrificed 24 hours following treatment. RNA isolated from these animals was also used for microarray and qRT-PCR analyses. The last set of animals was treated with 0.1 - 0.2 mM BSA and sacrificed 4, 24 or 46-50 hours following treatment for Smad-P immunodetection.

***In vitro* slice preparation:** Brain slices for the *in vitro* experiments were prepared using standard techniques (Pavlovsky, Browne et al. 2003; Seiffert, Dreier et al. 2004; Ivens, Kaufer et al. 2007). Slices were transferred to a recording chamber where they were incubated in aCSF containing BSA (0.1 mM), TGF-β1 (10 ng/ml) or artificial serum (aSERUM, composition based on aCSF with the following changes, composition in mM: 0.8 MgSO4, 1.3 CaCl2, 5.7 KCl, 1 L-glutamine, 0.1 BSA). To block the activity of TGF-β1, slices were incubated in aCSF containing SB431542 (10 μM) before the addition of TGF-β1 (10ng/ml). To block TGF-βRs, slices were incubated in aCSF containing SB431542 (10 μM) and TGF-βRII antibody (10 μg/ml) for 30 min followed by incubation in BSA in the presence of TGF-βR blockers. For detection of epileptiform activity, field

potentials were recorded 4-12 h following incubation in cortical layer IV using extracellular glass microelectrodes (~3MΩ) in response to bipolar stimulation at the border of white and grey matter. The time of recording was chosen based on the occurrence of epileptiform activity 4-8 hours following albumin exposure in the slice preparation (Ivens, Kaufer et al. 2007).

Albumin and TGF-βRII Co-Immunoprecipitation: To prepare cortical lysates, brains were isolated from naïve adult Wistar rats, dissected in cold saline solution and lysed in RIPA buffer. BSA (3 μg) was added to lysates to approximately match the amount of precipitating anti-albumin antibodies. Immunoprecipitation was performed using the Catch and Release® v2.0 Reversible Immunoprecipitation System (Upstate, Charlottesville, Virginia) with the following modifications to the standard protocol: the starting amount of protein was increased to 1,500 μg and the incubation time with precipitating antibodies was increased to 90 minutes. Lysate samples (positive or negative for albumin) were immunoprecipitated with an anti-TGF-βRII antibody (Upstate) or an anti-albumin antibody (Biogenesis, Poole, UK).

The immunoprecipitated samples were separated with SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was stained with Ponceau S stain to confirm that the IP procedure was successful. It was then destained, and blocked with 5% BSA in standard TBS-T buffer overnight at 4°C. TGF-βRII was detected with a rabbit anti-TGF-βRII antibody (Upstate) and an AP-conjugated donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Chemiluminescent detection was done using Lumi-Phos WB Chemiluminescent Substrate (Pierce, Rockfort, IL) and standard X-ray film according to the manufacturer's instructions.

Microarrays: Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA) and prepared using the Affymetrix GeneChip one-cycle target labeling kit (Affymetrix, Santa Clara, CA). Biotinylated cRNA was fragmented and hybridized to the GeneChip Rat Genome 230 2.0 Array according to company protocols (Affymetrix Technical Manual). Normalization of the array data was done using GCRMA (GC Robust Multi-Array Average) analysis, which takes into account the GC content of the probe sequences (Wu, Irizarry et al. 2004). Functional annotation analysis was performed with the program Database for Annotation, Visualization, and Integrated Discovery (DAVID) 2008 (Dennis, Sherman et al. 2003) (http://david.abcc.ncifcrf.gov). Unspecific (e.g., cellular process) and redundant terms (e.g., death and cell death) were removed and the full lists are provided in Supplementary Tables 1-8. The GenMAPP 2.0 program (Salomonis, Hanspers et al. 2007) (http://www.genmapp.org/) was used to visualize genes involved in TGF-β signaling.

For the time course analysis one array was run for each treatment (DOC, BSA, TGF-β1) for the following time points: 7/8, 24, and 48 hr. In addition a sample from a sham treated animal (24hr) was run and used to normalize the other arrays. Pairwise Pearson correlation coefficients for the three treatments were determined with Excel (Microsoft Corp., Richmod, WA). Hierarchical clustering was performed with Gene Cluster and displayed with TreeView software (Eisen, Spellman et al. 1998). To identify genes involved in astrocytic functions, we used GeneCards (http://www.genecards.org), querying for "astrocyte". For comparison of the relative changes in the expression of astrocytic vs. neuronal genes, gene sets published by Cahoy and colleagues (2008) were used to identify astrocytic and neuronal enriched genes (expressed by S100β+ and S100β-/PDGFRα-/MOG-cells, respectively).

Arrays were then run for the second set of animals sacrificed 24 hr following treatment (Sham, n=2; BSA, n=3; BSA + TGF-βR blockers, n=4). Significance analysis of microarrays (SAM) was performed with a false discovery rate (FDR) threshold of 9.2%. A 1.5 fold change cutoff was also used to filter this list. Genes from this filtered list, which demonstrated a log2 ratio difference > 0.5 between the albumin and albumin plus blocker treatments were considered part of the attenuated response. The remaining genes were considered part of the unattenuated response. All microarray data are available at the GEO website (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE12304.

Real-time RT-PCR: mRNA expression levels were determined by quantitative reverse transcriptase-PCR by real-time kinetic analysis with an iQ5 detection system (Bio-Rad, Hercules, CA). Real - time PCR data were analyzed using the PCR Miner program (Leonoudakis, Zhao et al. 2008). 18S mRNA levels were used as internal controls for variations in sample preparation.

In-vitro neuronal culture preparations: Primary neuronal cortical cultures were prepared from embryonic day 18 rats as reported previously (Kaufer, Ogle et al. 2004). Briefly, cells were dissociated with a papain solution for 20 min at 37°C. After the removal of the papain solution, the tissue was resuspended in growth medium [MEM with Earle's salts containing 2.5% B27 supplement, 0.1% mito serum extender, 5% fetal bovine serum (FBS), 20 mM glucose, and 5 mM L-glutamine] and dissociated by mechanical trituration. The cells were plated, and after 4 h *in vitro* the cell culture medium was replaced with neurobasal medium supplemented with 2% B27 supplement and 0.5 mM GlutaMAX™. The cells were maintained in 5% CO_2 at 37°C. After 7 days *in vitro*, cytosine arabinofuranoside (AraC) (10 μM) was added to the cultures. After 10 days *in vitro*, the cells were incubated with 0.2-0.4 mM albumin for 4 or 24 h at 37°C.

In-vitro astrocytic culture preparations: For astrocytic cultures, astrocytes were isolated from the cerebral cortices of P1-2 rat pups. Cells were dissociated with papain and mechanical trituration. The cells were cultured in high-glucose Dulbecco's modified eagle medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and in 5% CO2 (medium was replaced every 2-3 days). After 10 days *in vitro*, the cells were shaken at 37°C for 3 hr to remove microglia and passaged onto plates. Once the cells were confluent, the culture medium was replaced with serum-free high-glucose DMEM (containing 1% penicillin/streptomycin) for 18 h. The cells were then incubated in serum-free medium containing 0.2-0.4 mM albumin for 4 or 24 h at 37°C.

Immunocytochemistry: For immunostainings cells were washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 min. The cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min and washed in PBS. They were then incubated with 5% normal donkey serum in PBS for one hour at room temperature followed by overnight incubation at 4°C with either mouse anti-NeuN (1:1000; Chemicon, Temecula, CA) or mouse anti-GFAP (1:1000; Cell Signaling Technology, Beverly, MA). The cells were washed in PBS, incubated with donkey anti-mouse Cy3 (1:1000; Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature, and then counterstained with DAPI.

Immunohistochemistry: For immunofluorescence, sections were incubated with 5% normal donkey serum in TBS containing 0.3% Triton X-100 for one hour at room temperature followed by overnight incubation at 4°C with either mouse anti-NeuN (1:100) plus rabbit anti-Smad1P (1:500; Cell Signaling Technology), mouse anti-NeuN plus rabbit anti-Smad2P (1:500; Millipore), mouse anti-GFAP (1:500) plus rabbit anti-Smad1P, or mouse anti-GFAP plus rabbit anti-Smad2P. The sections were washed in TBS, incubated with donkey anti-mouse DyLight 488 (1:500; Jackson ImmunoResearch) plus donkey ant-rabbit Cy3 (1:500), and then counterstained with DAPI.

Western Blot Analysis: All lysate samples were separated by SDS-PAGE. Lysate samples from sham-operated controls and animals treated with BSA were transferred onto a nitrocellulose membrane while lysate samples from primary cultures were transferred onto a polyvinylidene fluoride (PVDF) membrane. For Alk1, Alk5 and TGFBR2 western blots, membranes were blocked with 5% nonfat milk for one hour at room temperature followed by incubation overnight at 4°C in primary antibody and incubation with a peroxidase-conjugated secondary antibody for 1 hr at room temperature. For phospho-Smad1 and phospho-Smad2 western blots, membranes were blocked with 5% nonfat milk overnight at 4°C followed by incubation in primary antibody for 48 hr at 4°C and incubation with a peroxidase-conjugated secondary antibody for 2 hr at room temperature. Immunoblots were then visualized using ECL Plus Western blotting detection reagents (Amersham Biosciences). β-actin expression levels were used as a loading control. Antibodies used are

as follows: anti-Alk1 (Santa Cruz Biotechnology), anti-Alk5 (Santa Cruz Biotechnology), anti-TGFBR2 (Santa Cruz Biotechnology), anti-phospho-Smad1 (Cell Signaling), anti-phospho-Smad2 (Cell Signaling), and anti-actin (Sigma).

Statistical Analyses: For the electrophysiological data, differences between treated and control slices were determined by the Mann-Whitney U test for two independent samples or the chi-square test using SPSS 13.0 (SPSS Inc., Chicago, IL). Linear regression analysis for the microarray data was performed with Graphpad Prism (GraphPad Software, Inc., San Diego, CA). PCR data were analyzed with an unpaired Student's t test ($p < 0.05$ was considered significant) in Excel (Microsoft) or with the relative expression software tool (REST) (Pfaffl 2001). REST determines significance of the group ratio results with a randomization test. $p < 0.05$ was taken as the level of statistical significance.

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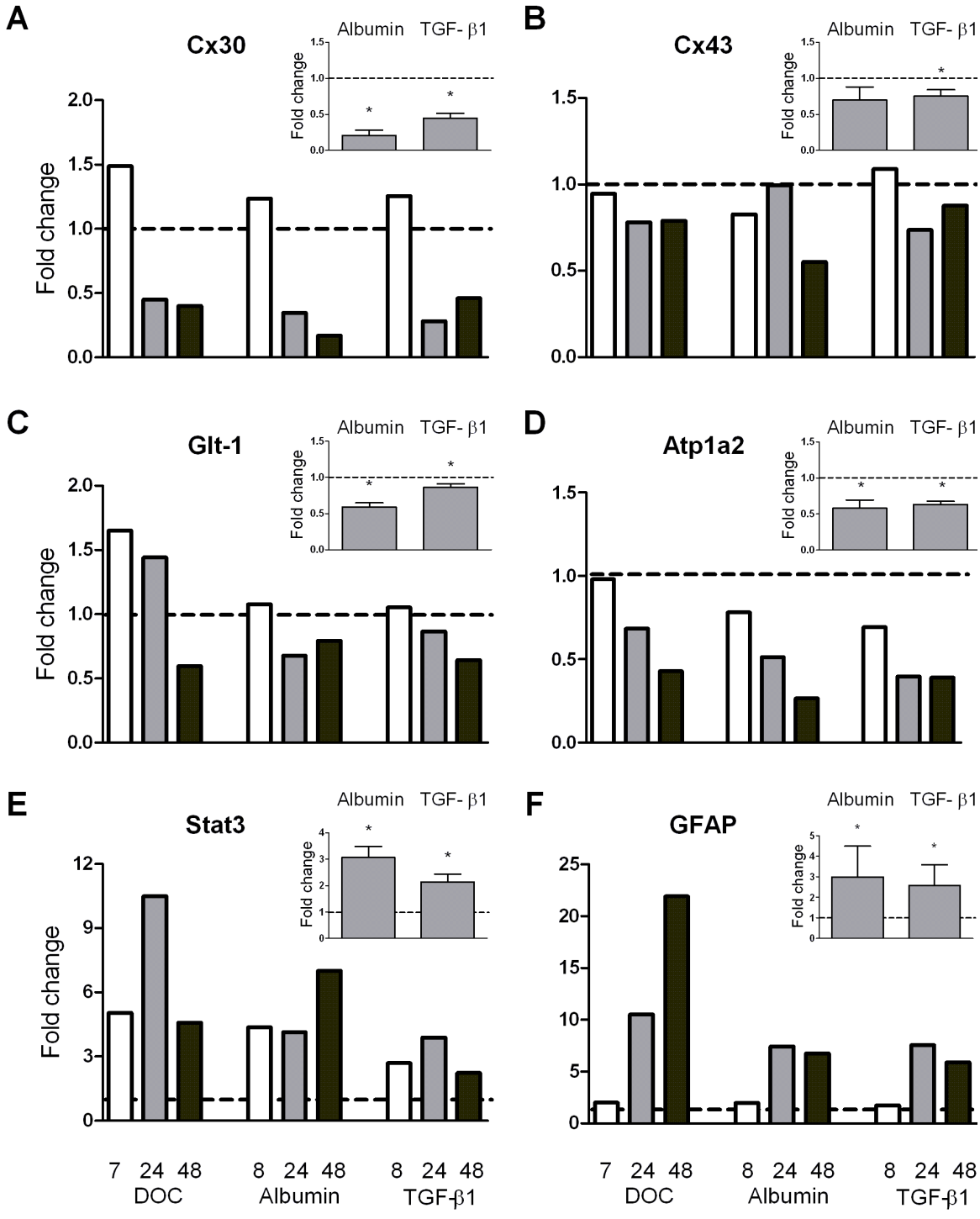
APPENDIX A


A
Cx30
Albumin TGF- β1
Fold change
B
Cx43
C
Glt-1
D
Atp1a2
E
Stat3
F
GFAP
7 24 48
DOC
8 24 48
Albumin
8 24 48
TGF-β1

Supplementary Figure 1. qRT-PCR gene expression analysis. Time course analysis for selected genes following treatment with DOC, albumin or TGF-β1 at 7/8, 24 or 48 hr. Data are expressed as fold changes relative to sham treated controls. Significance of changes was assessed for the 24 hour time point (shown in insets for albumin (n=3) and TGF-β1 (n=3) treatments; error bars indicate s.e.m. asterisks indicate $p<0.05$).

APPENDIX B

Supplementary Table 1: Full Gene Ontology list for genes downregulated during the early and middle time points following DOC, albumin or TGF-b treatments (BP = biological process, MF = molecular function).

Category	GO ID	Term	# genes	%	p-value
BP	GO:0019538	protein metabolic process	21	23.08%	4.45E-04
BP	GO:0044267	cellular protein metabolic process	20	21.98%	5.81E-04
BP	GO:0044260	cellular macromolecule metabolic process	20	21.98%	7.23E-04
MF	GO:0003824	catalytic activity	28	30.77%	1.28E-03
MF	GO:0004674	protein serine/threonine kinase activity	8	8.79%	1.37E-03
MF	GO:0004672	protein kinase activity	8	8.79%	5.05E-03
BP	GO:0043687	post-translational protein modification	10	10.99%	8.99E-03
MF	GO:0016773	phosphotransferase activity, alcohol group as acceptor	8	8.79%	1.09E-02
BP	GO:0006956	complement activation	3	3.30%	1.24E-02
BP	GO:0002541	activation of plasma proteins during acute inflammatory response	3	3.30%	1.24E-02
BP	GO:0006954	inflammatory response	5	5.49%	1.49E-02
MF	GO:0000166	nucleotide binding	13	14.29%	1.51E-02
BP	GO:0007242	intracellular signaling cascade	10	10.99%	1.56E-02
MF	GO:0004713	protein-tyrosine kinase activity	5	5.49%	1.58E-02
BP	GO:0009611	response to wounding	6	6.59%	1.61E-02
MF	GO:0005488	binding	40	43.96%	1.66E-02
BP	GO:0010038	response to metal ion	3	3.30%	1.97E-02
MF	GO:0016301	kinase activity	8	8.79%	2.14E-02
BP	GO:0002253	activation of immune response	3	3.30%	2.19E-02
BP	GO:0010035	response to inorganic substance	3	3.30%	2.30E-02

BP	GO:0006959	humoral immune response	3	3.30%	2.60E-02
BP	GO:0006464	protein modification process	10	10.99%	2.69E-02
BP	GO:0050778	positive regulation of immune response	3	3.30%	2.97E-02
MF	GO:0030554	adenyl nucleotide binding	10	10.99%	3.01E-02
BP	GO:0002684	positive regulation of immune system process	3	3.30%	3.16E-02
BP	GO:0043412	biopolymer modification	10	10.99%	3.24E-02
MF	GO:0005509	calcium ion binding	7	7.69%	3.53E-02
MF	GO:0017076	purine nucleotide binding	11	12.09%	3.76E-02
BP	GO:0043170	macromolecule metabolic process	24	26.37%	3.82E-02
MF	GO:0005529	sugar binding	4	4.40%	3.92E-02
BP	GO:0065007	biological regulation	19	20.88%	4.07E-02
BP	GO:0002526	acute inflammatory response	3	3.30%	4.12E-02
BP	GO:0050776	regulation of immune response	3	3.30%	4.12E-02
MF	GO:0016740	transferase activity	11	12.09%	4.13E-02
BP	GO:0002682	regulation of immune system process	3	3.30%	4.49E-02
MF	GO:0016772	transferase activity, transferring phosphorus-containing groups	8	8.79%	4.57E-02
BP	GO:0006468	protein amino acid phosphorylation	6	6.59%	4.69E-02

Supplementary Table 2: Full Gene Ontology list for genes upregulated during the early and middle time points following DOC, albumin or TGF-b treatments (BP = biological process, MF = molecular function).

Category	GO ID	Term	# genes	%	p-value
BP	GO:0048468	cell development	13	17.11%	1.03E-04
BP	GO:0030154	cell differentiation	14	18.42%	4.23E-04
BP	GO:0048869	cellular developmental process	14	18.42%	4.23E-04
BP	GO:0032502	developmental process	18	23.68%	0.00131
BP	GO:0065007	biological regulation	22	28.95%	0.00146
BP	GO:0048522	positive regulation of cellular process	10	13.16%	0.00158
BP	GO:0048518	positive regulation of biological process	10	13.16%	0.00307
BP	GO:0009605	response to external stimulus	8	10.53%	0.00416
BP	GO:0007243	protein kinase cascade	6	7.89%	0.00488
BP	GO:0016043	cellular component organization and biogenesis	15	19.74%	0.00546
BP	GO:0010001	glial cell differentiation	3	3.95%	0.00823
BP	GO:0050794	regulation of cellular process	17	22.37%	0.01002
BP	GO:0009967	positive regulation of signal transduction	4	5.26%	0.0103
BP	GO:0040007	growth	5	6.58%	0.01063
BP	GO:0042063	gliogenesis	3	3.95%	0.01081
BP	GO:0065008	regulation of biological quality	8	10.53%	0.01215
BP	GO:0007275	multicellular organismal development	13	17.11%	0.01288
BP	GO:0050789	regulation of biological process	18	23.68%	0.014
BP	GO:0006915	apoptosis	7	9.21%	0.01448
BP	GO:0012501	programmed cell death	7	9.21%	0.01536
BP	GO:0045595	regulation of cell differentiation	4	5.26%	0.01694
BP	GO:0044267	cellular protein metabolic process	16	21.05%	0.01707

BP	GO:0016265	death	7	9.21%	0.01792
BP	GO:0008219	cell death	7	9.21%	0.01792
BP	GO:0044260	cellular macromolecule metabolic process	16	21.05%	0.01977
BP	GO:0048731	system development	11	14.47%	0.02431
BP	GO:0048856	anatomical structure development	12	15.79%	0.02653
BP	GO:0048513	organ development	9	11.84%	0.02942
BP	GO:0019538	protein metabolic process	16	21.05%	0.03011
BP	GO:0044238	primary metabolic process	26	34.21%	0.03113
BP	GO:0008152	metabolic process	28	36.84%	0.03427
BP	GO:0051649	establishment of cellular localization	7	9.21%	0.03643
BP	GO:0051641	cellular localization	7	9.21%	0.03827
BP	GO:0009966	regulation of signal transduction	5	6.58%	0.04622

Supplementary Table 3: Full Gene Ontology list for genes downregulated during the middle and late time points following DOC, albumin or TGF-b treatments (BP = biological process, MF = molecular function).

Category	GO ID	Term	# genes	%	p-value
BP	GO:0007268	synaptic transmission	13	10.74%	1.71E-07
BP	GO:0019226	transmission of nerve impulse	13	10.74%	7.21E-07
BP	GO:0006810	transport	28	23.14%	1.12E-06
BP	GO:0051234	establishment of localization	28	23.14%	2.35E-06
BP	GO:0051179	localization	30	24.79%	2.66E-06
BP	GO:0051649	establishment of cellular localization	16	13.22%	4.38E-06
BP	GO:0051641	cellular localization	16	13.22%	5.06E-06
MF	GO:0005488	binding	62	51.24%	2.55E-05
BP	GO:0007267	cell-cell signaling	13	10.74%	5.67E-05
MF	GO:0005515	protein binding	41	33.88%	1.02E-04
MF	GO:0005215	transporter activity	17	14.05%	3.03E-04
MF	GO:0022836	gated channel activity	8	6.61%	3.26E-04
BP	GO:0016043	cellular component organization and biogenesis	23	19.01%	4.60E-04
BP	GO:0065008	regulation of biological quality	13	10.74%	4.63E-04
BP	GO:0007269	neurotransmitter secretion	5	4.13%	7.19E-04
MF	GO:0022892	substrate-specific transporter activity	14	11.57%	8.68E-04
BP	GO:0045055	regulated secretory pathway	5	4.13%	0.0012
MF	GO:0005216	ion channel activity	8	6.61%	0.00129
MF	GO:0005261	cation channel activity	7	5.79%	0.00144
MF	GO:0022838	substrate specific channel activity	8	6.61%	0.00155
MF	GO:0015267	channel activity	8	6.61%	0.00187
MF	GO:0022803	passive transmembrane transporter	8	6.61%	0.00187

		activity			
BP	GO:0065007	biological regulation	30	24.79%	0.00235
BP	GO:0030001	metal ion transport	8	6.61%	0.00246
MF	GO:0005234	extracellular-glutamate-gated ion channel activity	3	2.48%	0.00265
BP	GO:0046907	intracellular transport	10	8.26%	0.00303
BP	GO:0003001	generation of a signal involved in cell-cell signaling	5	4.13%	0.00329
MF	GO:0046873	metal ion transmembrane transporter activity	7	5.79%	0.00352
BP	GO:0001505	regulation of neurotransmitter levels	5	4.13%	0.00357
MF	GO:0004970	ionotropic glutamate receptor activity	3	2.48%	0.00374
MF	GO:0005230	extracellular ligand-gated ion channel activity	4	3.31%	0.00382
MF	GO:0008324	cation transmembrane transporter activity	9	7.44%	0.00406
BP	GO:0031326	regulation of cellular biosynthetic process	5	4.13%	0.00448
BP	GO:0006813	potassium ion transport	5	4.13%	0.00471
MF	GO:0022891	substrate-specific transmembrane transporter activity	11	9.09%	0.0058
MF	GO:0015075	ion transmembrane transporter activity	10	8.26%	0.00607
BP	GO:0006811	ion transport	10	8.26%	0.00609
BP	GO:0045045	secretory pathway	6	4.96%	0.0068
BP	GO:0007215	glutamate signaling pathway	3	2.48%	0.00685
BP	GO:0009889	regulation of biosynthetic process	5	4.13%	0.00767
BP	GO:0006812	cation transport	8	6.61%	0.00813
BP	GO:0046928	regulation of neurotransmitter secretion	3	2.48%	0.00841
BP	GO:0043062	extracellular structure organization	4	3.31%	0.01046

		and biogenesis			
MF	GO:0022834	ligand-gated channel activity	4	3.31%	0.01092
MF	GO:0015276	ligand-gated ion channel activity	4	3.31%	0.01092
MF	GO:0022857	transmembrane transporter activity	11	9.09%	0.01128
BP	GO:0032940	secretion by cell	6	4.96%	0.01319
BP	GO:0015672	monovalent inorganic cation transport	6	4.96%	0.01336
BP	GO:0048511	rhythmic process	4	3.31%	0.0136
BP	GO:0050789	regulation of biological process	25	20.66%	0.01557
MF	GO:0008565	protein transporter activity	4	3.31%	0.01579
BP	GO:0019216	regulation of lipid metabolic process	3	2.48%	0.01607
MF	GO:0019900	kinase binding	4	3.31%	0.01947
BP	GO:0031328	positive regulation of cellular biosynthetic process	3	2.48%	0.01988
MF	GO:0005267	potassium channel activity	4	3.31%	0.02359
BP	GO:0048167	regulation of synaptic plasticity	3	2.48%	0.02578
MF	GO:0000166	nucleotide binding	16	13.22%	0.02656
MF	GO:0022843	voltage-gated cation channel activity	4	3.31%	0.0303
BP	GO:0050803	regulation of synapse structure and activity	3	2.48%	0.03038
BP	GO:0009891	positive regulation of biosynthetic process	3	2.48%	0.03328
BP	GO:0046903	secretion	6	4.96%	0.03675
BP	GO:0009725	response to hormone stimulus	4	3.31%	0.03952
BP	GO:0019222	regulation of metabolic process	15	12.40%	0.04169
BP	GO:0040008	regulation of growth	4	3.31%	0.04261
MF	GO:0022832	voltage-gated channel activity	4	3.31%	0.04781
MF	GO:0005244	voltage-gated ion channel activity	4	3.31%	0.04781
MF	GO:0019899	enzyme binding	5	4.13%	0.04808

BP	GO:0008217	regulation of blood pressure	3	2.48%	0.04929

Supplementary Table 4: Full Gene Ontology list for genes upregulated during the middle and late time points following DOC, albumin or TGF-b treatments (BP = biological process, MF = molecular function).

Category	GO ID	Term	# genes	%	p-value
BP	GO:0008283	cell proliferation	21	12.07%	5.24E-06
BP	GO:0000278	mitotic cell cycle	12	6.90%	7.54E-06
MF	GO:0005515	protein binding	64	36.78%	9.26E-06
BP	GO:0007049	cell cycle	17	9.77%	1.11E-05
BP	GO:0022402	cell cycle process	15	8.62%	3.39E-05
BP	GO:0042127	regulation of cell proliferation	16	9.20%	3.47E-05
BP	GO:0051301	cell division	8	4.60%	7.86E-05
BP	GO:0006950	response to stress	23	13.22%	7.95E-05
BP	GO:0008285	negative regulation of cell proliferation	10	5.75%	1.77E-04
BP	GO:0009605	response to external stimulus	17	9.77%	3.95E-04
BP	GO:0000074	regulation of progression through cell cycle	10	5.75%	6.11E-04
BP	GO:0051726	regulation of cell cycle	10	5.75%	6.61E-04
MF	GO:0004866	endopeptidase inhibitor activity	7	4.02%	0.00131
BP	GO:0016043	cellular component organization and biogenesis	35	20.11%	0.00134
MF	GO:0030414	protease inhibitor activity	7	4.02%	0.0014
BP	GO:0045429	positive regulation of nitric oxide biosynthetic process	3	1.72%	0.00156
BP	GO:0007067	mitosis	7	4.02%	0.00161
MF	GO:0048306	calcium-dependent protein binding	3	1.72%	0.00171

MF	GO:0030234	enzyme regulator activity	14	8.05%	0.00182
BP	GO:0000279	M phase	8	4.60%	0.00184
BP	GO:0000087	M phase of mitotic cell cycle	7	4.02%	0.00185
BP	GO:0048518	positive regulation of biological process	20	11.49%	0.00187
BP	GO:0022403	cell cycle phase	9	5.17%	0.0023
BP	GO:0018193	peptidyl-amino acid modification	6	3.45%	0.0027
BP	GO:0051052	regulation of DNA metabolic process	4	2.30%	0.00271
BP	GO:0048519	negative regulation of biological process	19	10.92%	0.00291
BP	GO:0050789	regulation of biological process	45	25.86%	0.00305
BP	GO:0048523	negative regulation of cellular process	18	10.34%	0.00333
MF	GO:0004857	enzyme inhibitor activity	8	4.60%	0.00394
BP	GO:0045428	regulation of nitric oxide biosynthetic process	3	1.72%	0.00399
BP	GO:0006260	DNA replication	7	4.02%	0.00417
MF	GO:0004867	serine-type endopeptidase inhibitor activity	5	2.87%	0.006
MF	GO:0042802	identical protein binding	8	4.60%	0.00695
BP	GO:0045766	positive regulation of angiogenesis	3	1.72%	0.00744
BP	GO:0006275	regulation of DNA replication	3	1.72%	0.00744
BP	GO:0007242	intracellular signaling cascade	21	12.07%	0.00753
BP	GO:0065007	biological regulation	48	27.59%	0.0078
BP	GO:0032989	cellular structure morphogenesis	11	6.32%	0.00803
BP	GO:0000902	cell morphogenesis	11	6.32%	0.00803
BP	GO:0030154	cell differentiation	24	13.79%	0.00979
BP	GO:0048869	cellular developmental process	24	13.79%	0.00979
BP	GO:0045087	innate immune response	4	2.30%	0.01064
BP	GO:0006956	complement activation	4	2.30%	0.0118
BP	GO:0002541	activation of plasma proteins during acute	4	2.30%	0.0118

		inflammatory response			
MF	GO:0046982	protein heterodimerization activity	6	3.45%	0.01272
BP	GO:0050776	regulation of immune response	5	2.87%	0.01278
BP	GO:0046209	nitric oxide metabolic process	3	1.72%	0.01308
BP	GO:0006809	nitric oxide biosynthetic process	3	1.72%	0.01308
BP	GO:0048468	cell development	19	10.92%	0.01418
BP	GO:0002455	humoral immune response mediated by circulating immunoglobulin	3	1.72%	0.01438
BP	GO:0006958	complement activation, classical pathway	3	1.72%	0.01438
BP	GO:0048015	phosphoinositide-mediated signaling	5	2.87%	0.01453
BP	GO:0002682	regulation of immune system process	5	2.87%	0.01499
BP	GO:0040008	regulation of growth	6	3.45%	0.01616
BP	GO:0019538	protein metabolic process	39	22.41%	0.01628
BP	GO:0007088	regulation of mitosis	4	2.30%	0.01638
BP	GO:0007243	protein kinase cascade	9	5.17%	0.0176
BP	GO:0050794	regulation of cellular process	38	21.84%	0.0177
BP	GO:0006954	inflammatory response	8	4.60%	0.01786
BP	GO:0007010	cytoskeleton organization and biogenesis	10	5.75%	0.01857
MF	GO:0005184	neuropeptide hormone activity	3	1.72%	0.0194
BP	GO:0031667	response to nutrient levels	5	2.87%	0.01954
BP	GO:0001558	regulation of cell growth	5	2.87%	0.01954
BP	GO:0032502	developmental process	35	20.11%	0.0208
BP	GO:0009991	response to extracellular stimulus	5	2.87%	0.0224
MF	GO:0046983	protein dimerization activity	8	4.60%	0.02373
BP	GO:0009611	response to wounding	10	5.75%	0.02463
BP	GO:0002376	immune system process	13	7.47%	0.02578
BP	GO:0007249	I-kappaB kinase/NF-kappaB cascade	5	2.87%	0.02615

BP	GO:0002253	activation of immune response	4	2.30%	0.02626
MF	GO:0005488	binding	87	50.00%	0.02649
BP	GO:0044238	primary metabolic process	67	38.51%	0.02714
BP	GO:0051240	positive regulation of multicellular organismal process	5	2.87%	0.02748
BP	GO:0006259	DNA metabolic process	11	6.32%	0.02835
BP	GO:0006996	organelle organization and biogenesis	15	8.62%	0.03303
BP	GO:0051329	interphase of mitotic cell cycle	4	2.30%	0.0332
BP	GO:0006959	humoral immune response	4	2.30%	0.0332
BP	GO:0048002	antigen processing and presentation of peptide antigen	4	2.30%	0.0332
BP	GO:0010033	response to organic substance	5	2.87%	0.03396
BP	GO:0051325	interphase	4	2.30%	0.03426
BP	GO:0000910	cytokinesis	3	1.72%	0.03568
BP	GO:0019932	second-messenger-mediated signaling	7	4.02%	0.0362
MF	GO:0016301	kinase activity	13	7.47%	0.0369
BP	GO:0051239	regulation of multicellular organismal process	8	4.60%	0.03961
BP	GO:0050778	positive regulation of immune response	4	2.30%	0.03984
BP	GO:0042981	regulation of apoptosis	10	5.75%	0.04118
MF	GO:0031404	chloride ion binding	3	1.72%	0.04218
BP	GO:0040007	growth	7	4.02%	0.04299
BP	GO:0002684	positive regulation of immune system process	4	2.30%	0.04339
BP	GO:0016049	cell growth	5	2.87%	0.04387
MF	GO:0043168	anion binding	3	1.72%	0.04407
BP	GO:0044267	cellular protein metabolic process	35	20.11%	0.04444
BP	GO:0043067	regulation of programmed cell death	10	5.75%	0.0446

BP	GO:0009967	positive regulation of signal transduction	5	2.87%	0.04476
BP	GO:0007584	response to nutrient	4	2.30%	0.04585
MF	GO:0003824	catalytic activity	49	28.16%	0.04848
MF	GO:0032403	protein complex binding	4	2.30%	0.04911
BP	GO:0008152	metabolic process	72	41.38%	0.04915
BP	GO:0008361	regulation of cell size	5	2.87%	0.04937

Supplementary Table 5: Full Gene Ontology list for genes downregulated following treatment with albumin or albumin plus TGF-b receptor blockers (unattenuated response; BP = biological process, MF = molecular function).

Category	GO ID	Term	# genes	%	p-value
MF	GO:0016769	transferase activity, transferring nitrogenous groups	3	3.19%	0.00531
MF	GO:0048037	cofactor binding	5	5.32%	0.00762
MF	GO:0005488	binding	41	43.62%	0.01334
BP	GO:0007399	nervous system development	8	8.51%	0.01498
MF	GO:0005539	glycosaminoglycan binding	3	3.19%	0.02841
BP	GO:0048699	generation of neurons	5	5.32%	0.03077
MF	GO:0030247	polysaccharide binding	3	3.19%	0.03119
BP	GO:0048856	anatomical structure development	13	13.83%	0.03298
MF	GO:0001871	pattern binding	3	3.19%	0.03858
BP	GO:0032502	developmental process	16	17.02%	0.04072
MF	GO:0001664	G-protein-coupled receptor binding	3	3.19%	0.04171
BP	GO:0022008	neurogenesis	5	5.32%	0.04191
MF	GO:0005102	receptor binding	7	7.45%	0.04521
BP	GO:0048468	cell development	9	9.57%	0.04623

Supplementary Table 6: Full Gene Ontology list for genes upregulated following treatment with albumin or albumin plus TGF-b receptor blockers (unattenuated response; BP = biological process, MF = molecular function).

Category	GO ID	Term	# genes	%	p-value
MF	GO:0005488	binding	97	60.25%	3.50E-08
MF	GO:0045182	translation regulator activity	9	5.59%	7.78E-07
MF	GO:0008135	translation factor activity, nucleic acid binding	8	4.97%	4.05E-06
MF	GO:0005515	protein binding	62	38.51%	4.73E-06
BP	GO:0022618	protein-RNA complex assembly	7	4.35%	4.71E-05
BP	GO:0006413	translational initiation	6	3.73%	6.18E-05
MF	GO:0000166	nucleotide binding	29	18.01%	1.56E-04
BP	GO:0007242	intracellular signaling cascade	23	14.29%	2.26E-04
MF	GO:0032555	purine ribonucleotide binding	25	15.53%	4.03E-04
MF	GO:0032553	ribonucleotide binding	25	15.53%	4.03E-04
BP	GO:0016043	cellular component organization and biogenesis	33	20.50%	4.61E-04
MF	GO:0003743	translation initiation factor activity	5	3.11%	4.99E-04
BP	GO:0022613	ribonucleoprotein complex biogenesis and assembly	7	4.35%	5.95E-04
BP	GO:0007243	protein kinase cascade	11	6.83%	6.11E-04
MF	GO:0017076	purine nucleotide binding	25	15.53%	7.68E-04
MF	GO:0003924	GTPase activity	7	4.35%	0.00124
MF	GO:0016853	isomerase activity	6	3.73%	0.00221
BP	GO:0065003	macromolecular complex assembly	11	6.83%	0.00228
MF	GO:0016462	pyrophosphatase activity	12	7.45%	0.00296
MF	GO:0016772	transferase activity, transferring phosphorus-containing groups	17	10.56%	0.00309

MF	GO:0016818	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	12	7.45%	0.00313
MF	GO:0016817	hydrolase activity, acting on acid anhydrides	12	7.45%	0.00335
BP	GO:0022607	cellular component assembly	11	6.83%	0.00439
MF	GO:0016301	kinase activity	15	9.32%	0.00445
MF	GO:0003824	catalytic activity	51	31.68%	0.00595
BP	GO:0006916	anti-apoptosis	6	3.73%	0.00662
MF	GO:0017111	nucleoside-triphosphatase activity	11	6.83%	0.00675
BP	GO:0050789	regulation of biological process	39	24.22%	0.00723
BP	GO:0043066	negative regulation of apoptosis	7	4.35%	0.00738
BP	GO:0065007	biological regulation	43	26.71%	0.00751
BP	GO:0043069	negative regulation of programmed cell death	7	4.35%	0.0077
MF	GO:0032561	guanyl ribonucleotide binding	8	4.97%	0.00842
MF	GO:0019001	guanyl nucleotide binding	8	4.97%	0.00871
BP	GO:0019538	protein metabolic process	36	22.36%	0.00887
BP	GO:0006468	protein amino acid phosphorylation	12	7.45%	0.00933
BP	GO:0048519	negative regulation of biological process	16	9.94%	0.00979
BP	GO:0006928	cell motility	9	5.59%	0.01019
BP	GO:0051674	localization of cell	9	5.59%	0.01019
BP	GO:0050794	regulation of cellular process	35	21.74%	0.01031
BP	GO:0009112	nucleobase metabolic process	3	1.86%	0.01115
BP	GO:0016310	phosphorylation	13	8.07%	0.01188
BP	GO:0046907	intracellular transport	12	7.45%	0.01197
BP	GO:0048523	negative regulation of cellular process	15	9.32%	0.01236
MF	GO:0032559	adenyl ribonucleotide binding	18	11.18%	0.01328
BP	GO:0044260	cellular macromolecule metabolic	34	21.12%	0.0134

		process			
BP	GO:0006915	apoptosis	12	7.45%	0.01515
BP	GO:0009987	cellular process	93	57.76%	0.0164
BP	GO:0012501	programmed cell death	12	7.45%	0.01655
BP	GO:0044267	cellular protein metabolic process	33	20.50%	0.01849
BP	GO:0042981	regulation of apoptosis	10	6.21%	0.01938
BP	GO:0043170	macromolecule metabolic process	53	32.92%	0.02066
BP	GO:0008219	cell death	12	7.45%	0.02078
BP	GO:0016265	death	12	7.45%	0.02078
MF	GO:0030554	adenyl nucleotide binding	18	11.18%	0.0211
BP	GO:0043067	regulation of programmed cell death	10	6.21%	0.02115
BP	GO:0007264	small GTPase mediated signal transduction	8	4.97%	0.02131
MF	GO:0005524	ATP binding	17	10.56%	0.02354
BP	GO:0007010	cytoskeleton organization and biogenesis	9	5.59%	0.02362
MF	GO:0016773	phosphotransferase activity, alcohol group as acceptor	12	7.45%	0.02363
BP	GO:0008283	cell proliferation	12	7.45%	0.02488
MF	GO:0005525	GTP binding	7	4.35%	0.02562
BP	GO:0006446	regulation of translational initiation	3	1.86%	0.02947
BP	GO:0044238	primary metabolic process	59	36.65%	0.03308
BP	GO:0043687	post-translational protein modification	16	9.94%	0.03316
MF	GO:0003697	single-stranded DNA binding	3	1.86%	0.03321
BP	GO:0006412	translation	10	6.21%	0.03623
MF	GO:0016740	transferase activity	20	12.42%	0.03636
MF	GO:0004674	protein serine/threonine kinase activity	9	5.59%	0.03773
BP	GO:0051246	regulation of protein metabolic process	6	3.73%	0.03972
BP	GO:0006793	phosphorus metabolic process	13	8.07%	0.03985

BP	GO:0006796	phosphate metabolic process	13	8.07%	0.03985
BP	GO:0042127	regulation of cell proliferation	9	5.59%	0.04058
BP	GO:0046483	heterocycle metabolic process	4	2.48%	0.0437
BP	GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	5	3.11%	0.04652
BP	GO:0006937	regulation of muscle contraction	3	1.86%	0.04667
MF	GO:0003676	nucleic acid binding	27	16.77%	0.04781

Supplementary Table 7: Full Gene Ontology list for genes downregulated following treatment with albumin and attenuated following treatment with albumin plus TGF-b receptor blockers (attenuated response; BP = biological process, MF = molecular function).

Category	GO ID	Term	# genes	%	p-value
MF	GO:0016491	oxidoreductase activity	28	8.56%	9.55E-06
BP	GO:0007275	multicellular organismal development	51	15.60%	1.29E-05
BP	GO:0048856	anatomical structure development	49	14.98%	2.85E-05
BP	GO:0001501	skeletal development	13	3.98%	1.13E-04
BP	GO:0032502	developmental process	60	18.35%	1.16E-04
BP	GO:0009653	anatomical structure morphogenesis	30	9.17%	1.59E-04
MF	GO:0005507	copper ion binding	7	2.14%	2.58E-04
MF	GO:0019842	vitamin binding	8	2.45%	4.87E-04
BP	GO:0048731	system development	40	12.23%	6.08E-04
BP	GO:0007399	nervous system development	23	7.03%	6.77E-04
BP	GO:0006820	anion transport	10	3.06%	7.40E-04
BP	GO:0006810	transport	50	15.29%	7.45E-04
BP	GO:0006082	organic acid metabolic process	18	5.50%	9.03E-04
BP	GO:0006811	ion transport	22	6.73%	9.15E-04
BP	GO:0051234	establishment of localization	51	15.60%	9.41E-04
BP	GO:0048513	organ development	31	9.48%	0.00155
BP	GO:0006118	electron transport	15	4.59%	0.00182
BP	GO:0032787	monocarboxylic acid metabolic process	11	3.36%	0.00198
MF	GO:0043169	cation binding	49	14.98%	0.00213
BP	GO:0019752	carboxylic acid metabolic process	17	5.20%	0.00228
MF	GO:0003824	catalytic activity	81	24.77%	0.00231
MF	GO:0005215	transporter activity	29	8.87%	0.00239

BP	GO:0006576	biogenic amine metabolic process	6	1.83%	0.00309
BP	GO:0009790	embryonic development	12	3.67%	0.00319
BP	GO:0051179	localization	54	16.51%	0.00341
MF	GO:0046872	metal ion binding	50	15.29%	0.00358
BP	GO:0006817	phosphate transport	6	1.83%	0.00367
MF	GO:0022857	transmembrane transporter activity	22	6.73%	0.00459
MF	GO:0004497	monooxygenase activity	7	2.14%	0.00546
MF	GO:0043167	ion binding	50	15.29%	0.00581
BP	GO:0006584	catecholamine metabolic process	4	1.22%	0.00625
BP	GO:0040007	growth	11	3.36%	0.00634
MF	GO:0015075	ion transmembrane transporter activity	18	5.50%	0.00638
MF	GO:0022804	active transmembrane transporter activity	12	3.67%	0.00643
MF	GO:0004364	glutathione transferase activity	4	1.22%	0.00671
BP	GO:0018958	phenol metabolic process	4	1.22%	0.00691
MF	GO:0022891	substrate-specific transmembrane transporter activity	20	6.12%	0.00705
BP	GO:0006775	fat-soluble vitamin metabolic process	4	1.22%	0.0076
BP	GO:0001503	ossification	7	2.14%	0.01015
BP	GO:0031214	biomineral formation	7	2.14%	0.01015
BP	GO:0048598	embryonic morphogenesis	7	2.14%	0.01015
BP	GO:0006091	generation of precursor metabolites and energy	16	4.89%	0.01152
BP	GO:0044255	cellular lipid metabolic process	15	4.59%	0.01212
BP	GO:0006629	lipid metabolic process	17	5.20%	0.01215
BP	GO:0015698	inorganic anion transport	7	2.14%	0.01281
MF	GO:0005201	extracellular matrix structural constituent	5	1.53%	0.01323

MF	GO:0022892	substrate-specific transporter activity	22	6.73%	0.01329
BP	GO:0007565	female pregnancy	5	1.53%	0.01355
MF	GO:0050381	unspecific monooxygenase activity	4	1.22%	0.01358
MF	GO:0015294	solute:cation symporter activity	5	1.53%	0.01386
BP	GO:0006814	sodium ion transport	6	1.83%	0.01459
BP	GO:0001676	long-chain fatty acid metabolic process	3	0.92%	0.01494
MF	GO:0046914	transition metal ion binding	33	10.09%	0.01541
BP	GO:0006807	nitrogen compound metabolic process	13	3.98%	0.01646
BP	GO:0016049	cell growth	7	2.14%	0.01669
BP	GO:0006575	amino acid derivative metabolic process	6	1.83%	0.0175
MF	GO:0031402	sodium ion binding	5	1.53%	0.01874
BP	GO:0008361	regulation of cell size	7	2.14%	0.02001
BP	GO:0046849	bone remodeling	7	2.14%	0.02001
MF	GO:0005506	iron ion binding	9	2.75%	0.02244
BP	GO:0009888	tissue development	11	3.36%	0.02327
BP	GO:0007267	cell-cell signaling	17	5.20%	0.02374
MF	GO:0015293	symporter activity	6	1.83%	0.02378
BP	GO:0007160	cell-matrix adhesion	5	1.53%	0.02421
BP	GO:0030326	embryonic limb morphogenesis	4	1.22%	0.02427
BP	GO:0035113	embryonic appendage morphogenesis	4	1.22%	0.02427
MF	GO:0046943	carboxylic acid transmembrane transporter activity	5	1.53%	0.02548
BP	GO:0032989	cellular structure morphogenesis	13	3.98%	0.02562
BP	GO:0000902	cell morphogenesis	13	3.98%	0.02562
MF	GO:0005488	binding	133	40.67%	0.02566
BP	GO:0060173	limb development	4	1.22%	0.02568
BP	GO:0048736	appendage development	4	1.22%	0.02568

BP	GO:0035108	limb morphogenesis	4	1.22%	0.02568
BP	GO:0035107	appendage morphogenesis	4	1.22%	0.02568
BP	GO:0031589	cell-substrate adhesion	5	1.53%	0.02619
MF	GO:0005342	organic acid transmembrane transporter activity	5	1.53%	0.02642
BP	GO:0042417	dopamine metabolic process	3	0.92%	0.02686
BP	GO:0006766	vitamin metabolic process	5	1.53%	0.02722
MF	GO:0016712	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	4	1.22%	0.02726
BP	GO:0006519	amino acid and derivative metabolic process	11	3.36%	0.02794
BP	GO:0007155	cell adhesion	15	4.59%	0.02795
BP	GO:0022610	biological adhesion	15	4.59%	0.02795
BP	GO:0022414	reproductive process	9	2.75%	0.02799
MF	GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	6	1.83%	0.02819
BP	GO:0048771	tissue remodeling	7	2.14%	0.02821
BP	GO:0006631	fatty acid metabolic process	7	2.14%	0.02821
BP	GO:0048276	gastrulation (sensu Vertebrata)	3	0.92%	0.02958
BP	GO:0001702	gastrulation with mouth forming second	3	0.92%	0.02958
MF	GO:0015291	secondary active transmembrane transporter activity	7	2.14%	0.03034
BP	GO:0030900	forebrain development	4	1.22%	0.03172

MF	GO:0016765	transferase activity, transferring alkyl or aryl (other than methyl) groups	4	1.22%	0.03606
BP	GO:0001505	regulation of neurotransmitter levels	6	1.83%	0.03766
BP	GO:0006725	aromatic compound metabolic process	6	1.83%	0.0397
BP	GO:0019226	transmission of nerve impulse	12	3.67%	0.04048
BP	GO:0006812	cation transport	13	3.98%	0.04058
BP	GO:0008283	cell proliferation	17	5.20%	0.04096
BP	GO:0032501	multicellular organismal process	69	21.10%	0.04238
MF	GO:0004620	phospholipase activity	4	1.22%	0.04264
BP	GO:0007417	central nervous system development	8	2.45%	0.04346
BP	GO:0065007	biological regulation	65	19.88%	0.04639
BP	GO:0030001	metal ion transport	11	3.36%	0.04784

Supplementary Table 8: Full Gene Ontology list for genes upregulated following treatment with albumin and attenuated following treatment with albumin plus TGF-b receptor blockers (attenuated response; BP = biological process, MF = molecular function).

Category	GO ID	Term	# genes	%	p-value
MF	GO:0005515	protein binding	201	41.10%	5.15E-21
BP	GO:0009611	response to wounding	48	9.82%	2.14E-17
BP	GO:0006950	response to stress	72	14.72%	2.42E-17
BP	GO:0032502	developmental process	126	25.77%	1.51E-15

BP	GO:0065007	biological regulation	157	32.11%	2.67E-15
BP	GO:0050789	regulation of biological process	143	29.24%	8.22E-15
BP	GO:0009605	response to external stimulus	54	11.04%	1.52E-14
BP	GO:0048518	positive regulation of biological process	67	13.70%	4.20E-14
BP	GO:0048522	positive regulation of cellular process	62	12.68%	2.31E-13
BP	GO:0050794	regulation of cellular process	128	26.18%	3.64E-13
BP	GO:0006954	inflammatory response	32	6.54%	4.41E-12
BP	GO:0008219	cell death	49	10.02%	2.28E-11
BP	GO:0016265	death	49	10.02%	2.28E-11
BP	GO:0006928	cell motility	35	7.16%	2.84E-11
BP	GO:0051674	localization of cell	35	7.16%	2.84E-11
BP	GO:0048869	cellular developmental process	79	16.16%	4.42E-11
BP	GO:0030154	cell differentiation	79	16.16%	4.42E-11
BP	GO:0048519	negative regulation of biological process	59	12.07%	7.74E-11
BP	GO:0012501	programmed cell death	47	9.61%	8.30E-11
BP	GO:0006915	apoptosis	46	9.41%	1.83E-10
BP	GO:0009653	anatomical structure morphogenesis	59	12.07%	3.03E-10
BP	GO:0048523	negative regulation of cellular process	55	11.25%	4.07E-10
BP	GO:0048856	anatomical structure development	89	18.20%	4.67E-10
MF	GO:0005488	binding	271	55.42%	1.37E-09
BP	GO:0006952	defense response	35	7.16%	3.99E-09
BP	GO:0048513	organ development	63	12.88%	7.64E-09
BP	GO:0007275	multicellular organismal development	87	17.79%	1.04E-08
BP	GO:0016477	cell migration	26	5.32%	1.40E-08
BP	GO:0048731	system development	76	15.54%	1.95E-08
BP	GO:0043067	regulation of programmed cell death	36	7.36%	2.78E-08
BP	GO:0048468	cell development	59	12.07%	2.79E-08

BP	GO:0008283	cell proliferation	43	8.79%	5.36E-08
BP	GO:0042981	regulation of apoptosis	35	7.16%	6.27E-08
BP	GO:0050793	regulation of developmental process	24	4.91%	2.05E-07
BP	GO:0043066	negative regulation of apoptosis	21	4.29%	2.57E-07
BP	GO:0042127	regulation of cell proliferation	33	6.75%	2.68E-07
BP	GO:0007155	cell adhesion	36	7.36%	2.74E-07
BP	GO:0022610	biological adhesion	36	7.36%	2.74E-07
BP	GO:0043069	negative regulation of programmed cell death	21	4.29%	2.97E-07
BP	GO:0065008	regulation of biological quality	45	9.20%	5.40E-07
MF	GO:0046983	protein dimerization activity	26	5.32%	9.32E-07
BP	GO:0040007	growth	23	4.70%	1.22E-06
MF	GO:0003779	actin binding	19	3.89%	2.18E-06
BP	GO:0016043	cellular component organization and biogenesis	88	18.00%	2.21E-06
BP	GO:0007243	protein kinase cascade	26	5.32%	2.40E-06
BP	GO:0006916	anti-apoptosis	16	3.27%	3.00E-06
BP	GO:0065009	regulation of a molecular function	29	5.93%	3.89E-06
BP	GO:0007242	intracellular signaling cascade	56	11.45%	4.99E-06
BP	GO:0001944	vasculature development	17	3.48%	5.76E-06
BP	GO:0008285	negative regulation of cell proliferation	19	3.89%	6.38E-06
BP	GO:0009893	positive regulation of metabolic process	26	5.32%	6.67E-06
BP	GO:0048583	regulation of response to stimulus	10	2.04%	6.94E-06
MF	GO:0008092	cytoskeletal protein binding	24	4.91%	8.14E-06
BP	GO:0019222	regulation of metabolic process	72	14.72%	1.17E-05
BP	GO:0002376	immune system process	37	7.57%	1.48E-05
BP	GO:0001568	blood vessel development	16	3.27%	1.88E-05
BP	GO:0009607	response to biotic stimulus	17	3.48%	2.27E-05

MF	GO:0032403	protein complex binding	12	2.45%	2.71E-05
BP	GO:0042060	wound healing	15	3.07%	3.11E-05
BP	GO:0009887	organ morphogenesis	25	5.11%	3.62E-05
BP	GO:0007249	I-kappaB kinase/NF-kappaB cascade	13	2.66%	4.32E-05
BP	GO:0050727	regulation of inflammatory response	8	1.64%	4.62E-05
BP	GO:0031347	regulation of defense response	8	1.64%	4.62E-05
BP	GO:0050900	leukocyte migration	8	1.64%	4.62E-05
BP	GO:0031325	positive regulation of cellular metabolic process	23	4.70%	5.70E-05
MF	GO:0005539	glycosaminoglycan binding	10	2.04%	7.18E-05
BP	GO:0048514	blood vessel morphogenesis	14	2.86%	7.66E-05
BP	GO:0006366	transcription from RNA polymerase II promoter	29	5.93%	7.77E-05
BP	GO:0048646	anatomical structure formation	14	2.86%	8.72E-05
MF	GO:0042802	identical protein binding	19	3.89%	9.61E-05
BP	GO:0050790	regulation of catalytic activity	24	4.91%	9.72E-05
BP	GO:0000902	cell morphogenesis	26	5.32%	9.79E-05
BP	GO:0032989	cellular structure morphogenesis	26	5.32%	9.79E-05
BP	GO:0043065	positive regulation of apoptosis	18	3.68%	1.04E-04
BP	GO:0045935	positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	20	4.09%	1.05E-04
MF	GO:0030247	polysaccharide binding	10	2.04%	1.07E-04
BP	GO:0031323	regulation of cellular metabolic process	66	13.50%	1.07E-04
BP	GO:0009790	embryonic development	20	4.09%	1.10E-04
BP	GO:0043068	positive regulation of programmed cell death	18	3.68%	1.20E-04
BP	GO:0051179	localization	92	18.81%	1.57E-04

BP	GO:0045941	positive regulation of transcription	19	3.89%	1.67E-04
BP	GO:0001525	angiogenesis	12	2.45%	1.70E-04
BP	GO:0008284	positive regulation of cell proliferation	17	3.48%	1.82E-04
BP	GO:0006935	chemotaxis	11	2.25%	1.92E-04
BP	GO:0042330	taxis	11	2.25%	1.92E-04
BP	GO:0035239	tube morphogenesis	12	2.45%	2.10E-04
BP	GO:0009967	positive regulation of signal transduction	13	2.66%	2.26E-04
BP	GO:0051704	multi-organism process	16	3.27%	2.40E-04
MF	GO:0001871	pattern binding	10	2.04%	2.63E-04
BP	GO:0030005	cellular di-, tri-valent inorganic cation homeostasis	14	2.86%	2.70E-04
BP	GO:0055066	di-, tri-valent inorganic cation homeostasis	14	2.86%	2.70E-04
BP	GO:0051270	regulation of cell motility	9	1.84%	2.83E-04
BP	GO:0045637	regulation of myeloid cell differentiation	7	1.43%	2.98E-04
BP	GO:0051707	response to other organism	11	2.25%	3.00E-04
BP	GO:0008361	regulation of cell size	13	2.66%	3.05E-04
BP	GO:0055080	cation homeostasis	15	3.07%	3.16E-04
BP	GO:0030003	cellular cation homeostasis	15	3.07%	3.16E-04
BP	GO:0045859	regulation of protein kinase activity	15	3.07%	3.32E-04
BP	GO:0045595	regulation of cell differentiation	14	2.86%	3.53E-04
BP	GO:0043549	regulation of kinase activity	15	3.07%	4.45E-04
BP	GO:0016044	membrane organization and biogenesis	17	3.48%	4.82E-04
BP	GO:0045893	positive regulation of transcription, DNA-dependent	16	3.27%	5.02E-04
BP	GO:0040012	regulation of locomotion	9	1.84%	5.31E-04
BP	GO:0040008	regulation of growth	13	2.66%	5.64E-04
BP	GO:0030595	leukocyte chemotaxis	6	1.23%	5.83E-04

BP	GO:0051246	regulation of protein metabolic process	16	3.27%	5.94E-04
BP	GO:0051338	regulation of transferase activity	15	3.07%	6.15E-04
BP	GO:0040011	locomotion	9	1.84%	6.27E-04
MF	GO:0003690	double-stranded DNA binding	9	1.84%	6.29E-04
BP	GO:0009888	tissue development	20	4.09%	6.88E-04
BP	GO:0030334	regulation of cell migration	8	1.64%	6.88E-04
BP	GO:0051049	regulation of transport	12	2.45%	7.64E-04
BP	GO:0055065	metal ion homeostasis	12	2.45%	7.64E-04
BP	GO:0006875	cellular metal ion homeostasis	12	2.45%	7.64E-04
BP	GO:0016049	cell growth	12	2.45%	8.07E-04
BP	GO:0006357	regulation of transcription from RNA polymerase II promoter	22	4.50%	9.43E-04
BP	GO:0009617	response to bacterium	8	1.64%	9.92E-04
BP	GO:0035295	tube development	13	2.66%	1.04E-03
MF	GO:0051015	actin filament binding	6	1.23%	1.06E-03
BP	GO:0030036	actin cytoskeleton organization and biogenesis	13	2.66%	1.14E-03
BP	GO:0043085	positive regulation of catalytic activity	15	3.07%	1.27E-03
BP	GO:0006897	endocytosis	12	2.45%	1.30E-03
BP	GO:0010324	membrane invagination	12	2.45%	1.30E-03
BP	GO:0030099	myeloid cell differentiation	9	1.84%	1.34E-03
BP	GO:0055074	calcium ion homeostasis	11	2.25%	1.36E-03
BP	GO:0006874	cellular calcium ion homeostasis	11	2.25%	1.36E-03
MF	GO:0008201	heparin binding	7	1.43%	1.39E-03
BP	GO:0043123	positive regulation of I-kappaB kinase/ NF-kappaB cascade	8	1.64%	1.39E-03
BP	GO:0055082	cellular chemical homeostasis	15	3.07%	1.43E-03
BP	GO:0006873	cellular ion homeostasis	15	3.07%	1.43E-03

BP	GO:0001558	regulation of cell growth	10	2.04%	1.63E-03
BP	GO:0001816	cytokine production	9	1.84%	1.65E-03
MF	GO:0043566	structure-specific DNA binding	10	2.04%	1.74E-03
BP	GO:0030029	actin filament-based process	13	2.66%	1.79E-03
BP	GO:0002697	regulation of immune effector process	4	0.82%	1.79E-03
BP	GO:0048878	chemical homeostasis	17	3.48%	1.85E-03
BP	GO:0006606	protein import into nucleus	8	1.64%	1.91E-03
BP	GO:0051239	regulation of multicellular organismal process	19	3.89%	2.00E-03
BP	GO:0007626	locomotory behavior	12	2.45%	2.01E-03
BP	GO:0048534	hemopoietic or lymphoid organ development	13	2.66%	2.05E-03
BP	GO:0043122	regulation of I-kappaB kinase/ NF-kappaB cascade	8	1.64%	2.06E-03
BP	GO:0001775	cell activation	14	2.86%	2.10E-03
BP	GO:0002009	morphogenesis of an epithelium	9	1.84%	2.15E-03
MF	GO:0000166	nucleotide binding	62	12.68%	2.18E-03
BP	GO:0001655	urogenital system development	7	1.43%	2.21E-03
BP	GO:0051170	nuclear import	8	1.64%	2.22E-03
MF	GO:0019955	cytokine binding	8	1.64%	2.22E-03
BP	GO:0009966	regulation of signal transduction	22	4.50%	2.43E-03
BP	GO:0010468	regulation of gene expression	57	11.66%	2.55E-03
BP	GO:0006913	nucleocytoplasmic transport	10	2.04%	2.71E-03
BP	GO:0002520	immune system development	13	2.66%	2.74E-03
BP	GO:0019219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	55	11.25%	2.74E-03
BP	GO:0002526	acute inflammatory response	9	1.84%	2.75E-03
BP	GO:0019221	cytokine and chemokine mediated	6	1.23%	2.77E-03

		signaling pathway			
MF	GO:0042803	protein homodimerization activity	11	2.25%	2.95E-03
MF	GO:0019865	immunoglobulin binding	4	0.82%	3.00E-03
BP	GO:0042089	cytokine biosynthetic process	7	1.43%	3.11E-03
BP	GO:0006996	organelle organization and biogenesis	36	7.36%	3.28E-03
BP	GO:0051169	nuclear transport	10	2.04%	3.35E-03
BP	GO:0046651	lymphocyte proliferation	7	1.43%	3.37E-03
BP	GO:0032943	mononuclear cell proliferation	7	1.43%	3.37E-03
BP	GO:0042107	cytokine metabolic process	7	1.43%	3.37E-03
MF	GO:0005178	integrin binding	6	1.23%	3.41E-03
BP	GO:0050801	ion homeostasis	15	3.07%	3.46E-03
BP	GO:0009792	embryonic development ending in birth or egg hatching	10	2.04%	3.70E-03
BP	GO:0007610	behavior	17	3.48%	4.58E-03
BP	GO:0006275	regulation of DNA replication	4	0.82%	4.61E-03
MF	GO:0035091	phosphoinositide binding	9	1.84%	4.87E-03
BP	GO:0017038	protein import	8	1.64%	4.96E-03
BP	GO:0008156	negative regulation of DNA replication	3	0.61%	5.15E-03
BP	GO:0007568	aging	6	1.23%	5.45E-03
BP	GO:0048762	mesenchymal cell differentiation	5	1.02%	5.51E-03
BP	GO:0014031	mesenchymal cell development	5	1.02%	5.51E-03
BP	GO:0051235	maintenance of localization	5	1.02%	5.51E-03
MF	GO:0019838	growth factor binding	7	1.43%	5.69E-03
MF	GO:0016758	transferase activity, transferring hexosyl groups	10	2.04%	5.72E-03
BP	GO:0030198	extracellular matrix organization and biogenesis	6	1.23%	5.94E-03

MF	GO:0004907	interleukin receptor activity	5	1.02%	6.19E-03
BP	GO:0045321	leukocyte activation	12	2.45%	6.47E-03
MF	GO:0005102	receptor binding	30	6.13%	6.91E-03
BP	GO:0042592	homeostatic process	20	4.09%	6.91E-03
BP	GO:0033002	muscle cell proliferation	5	1.02%	6.91E-03
BP	GO:0006917	induction of apoptosis	12	2.45%	6.93E-03
BP	GO:0001822	kidney development	6	1.23%	7.02E-03
BP	GO:0012502	induction of programmed cell death	12	2.45%	7.18E-03
BP	GO:0019725	cellular homeostasis	16	3.27%	7.36E-03
BP	GO:0051094	positive regulation of developmental process	8	1.64%	7.44E-03
MF	GO:0019864	IgG binding	3	0.61%	7.61E-03
BP	GO:0031099	regeneration	7	1.43%	7.93E-03
BP	GO:0022402	cell cycle process	20	4.09%	8.13E-03
BP	GO:0001666	response to hypoxia	8	1.64%	8.29E-03
MF	GO:0019965	interleukin binding	5	1.02%	8.53E-03
BP	GO:0045449	regulation of transcription	51	10.43%	9.02E-03
BP	GO:0045944	positive regulation of transcription from RNA polymerase II promoter	11	2.25%	9.15E-03
MF	GO:0015020	glucuronosyltransferase activity	4	0.82%	9.17E-03
BP	GO:0045807	positive regulation of endocytosis	4	0.82%	9.17E-03
BP	GO:0030593	neutrophil chemotaxis	4	0.82%	9.17E-03
BP	GO:0006417	regulation of translation	9	1.84%	9.22E-03
BP	GO:0051241	negative regulation of multicellular organismal process	5	1.02%	9.42E-03
BP	GO:0009892	negative regulation of metabolic process	17	3.48%	9.56E-03
BP	GO:0030097	hemopoiesis	11	2.25%	1.02E-02

MF	GO:0003700	transcription factor activity	31	6.34%	1.04E-02
BP	GO:0030100	regulation of endocytosis	5	1.02%	1.04E-02
MF	GO:0019763	immunoglobulin receptor activity	3	0.61%	1.05E-02
BP	GO:0043009	chordate embryonic development	9	1.84%	1.05E-02
BP	GO:0045860	positive regulation of protein kinase activity	9	1.84%	1.05E-02
BP	GO:0050729	positive regulation of inflammatory response	4	0.82%	1.06E-02
BP	GO:0031349	positive regulation of defense response	4	0.82%	1.06E-02
BP	GO:0051092	activation of NF-kappaB transcription factor	4	0.82%	1.06E-02
BP	GO:0000074	regulation of progression through cell cycle	14	2.86%	1.07E-02
BP	GO:0051726	regulation of cell cycle	14	2.86%	1.16E-02
BP	GO:0007399	nervous system development	29	5.93%	1.19E-02
BP	GO:0033674	positive regulation of kinase activity	9	1.84%	1.25E-02
BP	GO:0051248	negative regulation of protein metabolic process	7	1.43%	1.28E-02
BP	GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	79	16.16%	1.32E-02
BP	GO:0043086	negative regulation of catalytic activity	7	1.43%	1.35E-02
BP	GO:0042246	tissue regeneration	6	1.23%	1.36E-02
BP	GO:0006939	smooth muscle contraction	5	1.02%	1.36E-02
BP	GO:0051100	negative regulation of binding	3	0.61%	1.38E-02
BP	GO:0002889	regulation of immunoglobulin mediated immune response	3	0.61%	1.38E-02
BP	GO:0002712	regulation of B cell mediated immunity	3	0.61%	1.38E-02
BP	GO:0002683	negative regulation of immune system process	3	0.61%	1.38E-02
MF	GO:0016563	transcription activator activity	14	2.86%	1.38E-02

MF	GO:0030246	carbohydrate binding	15	3.07%	1.40E-02
BP	GO:0009725	response to hormone stimulus	10	2.04%	1.41E-02
BP	GO:0048589	developmental growth	7	1.43%	1.42E-02
BP	GO:0051090	regulation of transcription factor activity	5	1.02%	1.48E-02
BP	GO:0045765	regulation of angiogenesis	5	1.02%	1.48E-02
MF	GO:0003677	DNA binding	49	10.02%	1.51E-02
BP	GO:0051347	positive regulation of transferase activity	9	1.84%	1.54E-02
BP	GO:0050670	regulation of lymphocyte proliferation	5	1.02%	1.61E-02
BP	GO:0032944	regulation of mononuclear cell proliferation	5	1.02%	1.61E-02
BP	GO:0006955	immune response	21	4.29%	1.70E-02
BP	GO:0009889	regulation of biosynthetic process	10	2.04%	1.74E-02
BP	GO:0042345	regulation of NF-kappaB import into nucleus	3	0.61%	1.74E-02
BP	GO:0002883	regulation of hypersensitivity	3	0.61%	1.74E-02
BP	GO:0002864	regulation of acute inflammatory response to antigenic stimulus	3	0.61%	1.74E-02
BP	GO:0002861	regulation of inflammatory response to antigenic stimulus	3	0.61%	1.74E-02
BP	GO:0002822	regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	3	0.61%	1.74E-02
BP	GO:0002819	regulation of adaptive immune response	3	0.61%	1.74E-02
BP	GO:0002706	regulation of lymphocyte mediated immunity	3	0.61%	1.74E-02
BP	GO:0002703	regulation of leukocyte mediated immunity	3	0.61%	1.74E-02

BP	GO:0051050	positive regulation of transport	6	1.23%	1.75E-02
BP	GO:0045767	regulation of anti-apoptosis	4	0.82%	1.76E-02
BP	GO:0010467	gene expression	73	14.93%	1.78E-02
MF	GO:0005543	phospholipid binding	10	2.04%	1.81E-02
BP	GO:0006350	transcription	52	10.63%	1.83E-02
BP	GO:0043405	regulation of MAP kinase activity	7	1.43%	1.84E-02
BP	GO:0044238	primary metabolic process	166	33.95%	1.90E-02
BP	GO:0008152	metabolic process	183	37.42%	1.95E-02
BP	GO:0045596	negative regulation of cell differentiation	6	1.23%	1.97E-02
BP	GO:0009719	response to endogenous stimulus	17	3.48%	2.00E-02
BP	GO:0007010	cytoskeleton organization and biogenesis	19	3.89%	2.00E-02
BP	GO:0031326	regulation of cellular biosynthetic process	9	1.84%	2.01E-02
MF	GO:0017076	purine nucleotide binding	51	10.43%	2.03E-02
BP	GO:0007167	enzyme linked receptor protein signaling pathway	13	2.66%	2.03E-02
BP	GO:0003012	muscle system process	9	1.84%	2.09E-02
BP	GO:0006936	muscle contraction	9	1.84%	2.09E-02
BP	GO:0045597	positive regulation of cell differentiation	6	1.23%	2.09E-02
BP	GO:0050896	response to stimulus	95	19.43%	2.10E-02
BP	GO:0022607	cellular component assembly	20	4.09%	2.12E-02
BP	GO:0051053	negative regulation of DNA metabolic process	3	0.61%	2.15E-02
BP	GO:0002673	regulation of acute inflammatory response	3	0.61%	2.15E-02
BP	GO:0002524	hypersensitivity	3	0.61%	2.15E-02
BP	GO:0002438	acute inflammatory response to antigenic stimulus	3	0.61%	2.15E-02

BP	GO:0045646	regulation of erythrocyte differentiation	3	0.61%	2.15E-02
BP	GO:0042348	NF-kappaB import into nucleus	3	0.61%	2.15E-02
BP	GO:0016331	morphogenesis of embryonic epithelium	5	1.02%	2.18E-02
MF	GO:0016757	transferase activity, transferring glycosyl groups	11	2.25%	2.19E-02
MF	GO:0043565	sequence-specific DNA binding	17	3.48%	2.29E-02
BP	GO:0006261	DNA-dependent DNA replication	6	1.23%	2.34E-02
MF	GO:0016301	kinase activity	30	6.13%	2.38E-02
BP	GO:0051091	positive regulation of transcription factor activity	4	0.82%	2.42E-02
BP	GO:0008629	induction of apoptosis by intracellular signals	4	0.82%	2.42E-02
MF	GO:0004672	protein kinase activity	24	4.91%	2.56E-02
BP	GO:0046649	lymphocyte activation	9	1.84%	2.58E-02
BP	GO:0006911	phagocytosis, engulfment	3	0.61%	2.58E-02
BP	GO:0002478	antigen processing and presentation of exogenous peptide antigen	3	0.61%	2.58E-02
BP	GO:0009628	response to abiotic stimulus	11	2.25%	2.67E-02
BP	GO:0043062	extracellular structure organization and biogenesis	7	1.43%	2.67E-02
MF	GO:0005201	extracellular matrix structural constituent	6	1.23%	2.74E-02
BP	GO:0006909	phagocytosis	4	0.82%	2.92E-02
BP	GO:0019932	second-messenger-mediated signaling	13	2.66%	3.03E-02
BP	GO:0050766	positive regulation of phagocytosis	3	0.61%	3.05E-02
MF	GO:0003924	GTPase activity	9	1.84%	3.15E-02
MF	GO:0032555	purine ribonucleotide binding	48	9.82%	3.18E-02
MF	GO:0032553	ribonucleotide binding	48	9.82%	3.18E-02

BP	GO:0048660	regulation of smooth muscle cell proliferation	4	0.82%	3.19E-02
BP	GO:0007050	cell cycle arrest	5	1.02%	3.25E-02
BP	GO:0065003	macromolecular complex assembly	18	3.68%	3.28E-02
BP	GO:0048545	response to steroid hormone stimulus	7	1.43%	3.31E-02
BP	GO:0007049	cell cycle	20	4.09%	3.40E-02
BP	GO:0042692	muscle cell differentiation	5	1.02%	3.45E-02
BP	GO:0042254	ribosome biogenesis and assembly	5	1.02%	3.45E-02
BP	GO:0022604	regulation of cell morphogenesis	4	0.82%	3.47E-02
BP	GO:0022603	regulation of anatomical structure morphogenesis	4	0.82%	3.47E-02
BP	GO:0048659	smooth muscle cell proliferation	4	0.82%	3.47E-02
BP	GO:0008360	regulation of cell shape	4	0.82%	3.47E-02
BP	GO:0008064	regulation of actin polymerization and/or depolymerization	4	0.82%	3.47E-02
BP	GO:0000003	reproduction	19	3.89%	3.49E-02
BP	GO:0016337	cell-cell adhesion	12	2.45%	3.56E-02
BP	GO:0007178	transmembrane receptor protein serine/threonine kinase signaling pathway	6	1.23%	3.68E-02
BP	GO:0031324	negative regulation of cellular metabolic process	14	2.86%	3.69E-02
MF	GO:0005125	cytokine activity	11	2.25%	3.70E-02
BP	GO:0030218	erythrocyte differentiation	4	0.82%	3.76E-02
BP	GO:0042311	vasodilation	4	0.82%	3.76E-02
BP	GO:0030832	regulation of actin filament length	4	0.82%	3.76E-02
BP	GO:0001838	embryonic epithelial tube formation	4	0.82%	3.76E-02
BP	GO:0051052	regulation of DNA metabolic process	4	0.82%	3.76E-02

BP	GO:0022613	ribonucleoprotein complex biogenesis and assembly	8	1.64%	3.84E-02
BP	GO:0048598	embryonic morphogenesis	8	1.64%	3.84E-02
BP	GO:0007204	elevation of cytosolic calcium ion concentration	6	1.23%	3.86E-02
BP	GO:0007179	transforming growth factor beta receptor signaling pathway	5	1.02%	3.88E-02
MF	GO:0016773	phosphotransferase activity, alcohol group as acceptor	26	5.32%	3.96E-02
BP	GO:0002682	regulation of immune system process	7	1.43%	4.02E-02
MF	GO:0004197	cysteine-type endopeptidase activity	7	1.43%	4.03E-02
BP	GO:0032535	regulation of cellular component size	4	0.82%	4.07E-02
MF	GO:0005024	transforming growth factor beta receptor activity	3	0.61%	4.08E-02
MF	GO:0004675	transmembrane receptor protein serine/threonine kinase activity	3	0.61%	4.08E-02
BP	GO:0045185	maintenance of protein localization	3	0.61%	4.09E-02
BP	GO:0050764	regulation of phagocytosis	3	0.61%	4.09E-02
BP	GO:0002437	inflammatory response to antigenic stimulus	3	0.61%	4.09E-02
BP	GO:0042990	regulation of transcription factor import into nucleus	3	0.61%	4.09E-02
BP	GO:0000165	MAPKKK cascade	9	1.84%	4.16E-02
BP	GO:0006979	response to oxidative stress	7	1.43%	4.18E-02
BP	GO:0033673	negative regulation of kinase activity	5	1.02%	4.34E-02
BP	GO:0006469	negative regulation of protein kinase activity	5	1.02%	4.34E-02
BP	GO:0010033	response to organic substance	8	1.64%	4.38E-02

MF	GO:0042379	chemokine receptor binding	4	0.82%	4.39E-02
MF	GO:0008009	chemokine activity	4	0.82%	4.39E-02
BP	GO:0045445	myoblast differentiation	4	0.82%	4.39E-02
BP	GO:0032956	regulation of actin cytoskeleton organization and biogenesis	4	0.82%	4.39E-02
BP	GO:0045165	cell fate commitment	6	1.23%	4.41E-02
BP	GO:0031589	cell-substrate adhesion	6	1.23%	4.41E-02
BP	GO:0051480	cytosolic calcium ion homeostasis	6	1.23%	4.41E-02
BP	GO:0009058	biosynthetic process	43	8.79%	4.43E-02
BP	GO:0032496	response to lipopolysaccharide	3	0.61%	4.64E-02
BP	GO:0002444	myeloid leukocyte mediated immunity	3	0.61%	4.64E-02
BP	GO:0007507	heart development	7	1.43%	4.67E-02
MF	GO:0003702	RNA polymerase II transcription factor activity	9	1.84%	4.67E-02
BP	GO:0030278	regulation of ossification	4	0.82%	4.72E-02
BP	GO:0006953	acute-phase response	4	0.82%	4.72E-02
MF	GO:0005525	GTP binding	13	2.66%	4.74E-02
BP	GO:0051348	negative regulation of transferase activity	5	1.02%	4.82E-02