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S100a9 Protects Against the Effects of Repeated Social Defeat Stress

Cassandra M. Moshfegh, Safwan K. Elkhatib, Gabrielle F. Watson, John Drake, Zachary N. Taylor, Emily C. Reed, Tatlock H. Lauten, Amelia J. Clopp, Vladimir I. Vladimirov, and Adam J. Case

ABSTRACT

BACKGROUND: Posttraumatic stress disorder, a consequence of psychological trauma, is associated with increased inflammation and an elevated risk of developing comorbid inflammatory diseases. However, the mechanistic link between this mental health disorder and inflammation remains elusive. We previously found that S100a8 and S100a9 messenger RNA, genes that encode the protein calprotectin, were significantly upregulated in T lymphocytes and positively correlated with inflammatory gene expression and the mitochondrial redox environment in these cells. Therefore, we hypothesized that genetic deletion of calprotectin would attenuate the inflammatory and redox phenotype displayed after psychological trauma.

METHODS: We used a preclinical mouse model of posttraumatic stress disorder known as repeated social defeat stress (RSDS) combined with pharmacological and genetic manipulation of S100a9 (which functionally eliminates calprotectin). A total of 186 animals (93 control, 93 RSDS) were used in these studies.

RESULTS: Unexpectedly, we observed worsening of behavioral pathology, inflammation, and the mitochondrial redox environment in mice after RSDS compared with wild-type animals. Furthermore, loss of calprotectin significantly enhanced the metabolic demand on T lymphocytes, suggesting that this protein may play an undescribed role in mitochondrial regulation. This was further supported by single-cell RNA sequencing analysis demonstrating that RSDS and loss of S100a9 primarily altered genes associated with mitochondrial function and oxidative phosphorylation.

CONCLUSIONS: These data demonstrate that the loss of calprotectin potentiates the RSDS-induced phenotype, which suggests that its observed upregulation after psychological trauma may provide previously unexplored protective functions.

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Posttraumatic stress disorder (PTSD), an illness characterized by behavioral pathology such as withdrawal, learned helplessness, hyperarousal, and flashbacks, affects nearly 45 million Americans (1–5). Individuals diagnosed with PTSD show significantly elevated risks for the development of comorbid inflammatory pathologies such as autoimmune, metabolic, and cardiovascular diseases (6–16). Inflammation is often observed in individuals with PTSD (13–18), and this may underlie this inherent risk for comorbid inflammatory diseases after PTSD. However, the mechanistic link between psychological trauma and altered immune function remains unknown and understudied.

Previous work from our laboratory identified a significant elevation in 2 inflammatory calcium-binding proteins, S100a8 (calgranulin A) and S100a9 (calgranulin B), in T lymphocytes, but not other immune cells, in a mouse model of psychological trauma (i.e., repeated social defeat stress [RSDS]) (19). Together, these proteins form the heterodimeric proinflammatory protein calprotectin, which has been extensively studied within the innate immune system but had not been reported in T lymphocytes until our previous work (19). Canonically, calprotectin acts as a damage-associated molecular pattern while also sequestering ions such as iron, manganese, zinc, and calcium to inhibit pathogen growth (20–23). Interestingly, extracellular calprotectin has been shown to enhance the development of autoreactive $CD8^+$ T lymphocytes and enhanced interleukin 17A (IL-17A) production in T lymphocytes (24) and has been implicated in numerous autoimmune diseases (22,25–28). Furthermore, calprotectin is known to be redox regulated and plays a critical role in intracellular redox signaling (29). Given that we previously demonstrated that calprotectin was correlated with behavioral pathology, inflammation, and redox changes after RSDS (19), these data strongly suggest that calprotectin may play a mechanistic role in potentiating the proinflammatory T lymphocyte phenotype that we and others have observed after RSDS (19,30–32).

In the present study, we hypothesized that loss of calprotectin would attenuate the pathology associated with RSDS.

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To test this hypothesis, we investigated the behavioral, inflammatory, redox, metabolic, and gene expression changes of RSDS in S100a9 knockout (S100a9$^{-/-}$) mice, which lack functional calprotectin. Surprisingly, and in contrast to our hypothesis, we observed that loss of S100a9 exacerbated circulating and T-lymphocyte inflammation and worsened specific behaviors after RSDS. Moreover, the mitochondrial redox and metabolic environments of S100a9$^{-/-}$ T lymphocytes were significantly perturbed compared with wild-type (WT) RSDS mice. Single-cell RNA sequencing analysis on S100a9$^{-/-}$ T lymphocytes showed that loss of S100a9 had significant impacts on genes regulating mitochondrial function and oxidative phosphorylation, suggesting a significant mitochondrial role of S100a9 in T lymphocytes. Together, these data show for the first time a functional role of S100a9 in T lymphocytes that may be protective in attenuating phenotypic aspects of psychological trauma.

METHODS AND MATERIALS

WT C57BL/6J mice were obtained from Jackson Laboratories (#000664). S100a9$^{-/-}$ mice were obtained from the Mutant Mouse Resource and Research Centers at the University of California Davis (#049540). CD1 male retired breeder mice were purchased from Charles River at age 4 to 6 months (#022). A total of 186 animals (93 control, 93 RSDS) were used in these studies.

For detailed methods and materials, see the Supplement.

RESULTS

S100a8 and S100a9 Messenger RNA and Protein Are Elevated in T Lymphocytes After RSDS

Previous work from our laboratory demonstrated that calprotectin was elevated over 3-fold in circulation of RSDS mice, and, to our knowledge, we were the first to report its presence in T lymphocytes using single-cell RNA sequencing (19). Herein, we again validate that socially defeated adult male mice consistently show elevations in S100a8 and S100a9 messenger RNA (mRNA) transcript levels in splenic T lymphocytes and extend these findings to demonstrate that removal of the splenic nerve (i.e., denervation) fully attenuates this induction in T lymphocytes (Figure 1A). Interestingly, while blood immune cells also demonstrated an increase in S100a8 and S100a9 mRNA after RSDS, immune cells from lymph nodes (inguinal and mesenteric) did not (Figure S1A). For this reason, we chose to examine splenic T lymphocytes in our in-depth analyses moving forward. Furthermore, we confirmed that adult RSDS mice possess intracellular S100a8 and S100a9 protein in T lymphocytes and that these proteins significantly correlate with each other within these cells (r = 0.9827, $p < .0001$) (Figure 1B). While knockout of S100a9 caused the expected loss of S100a9 within T lymphocytes, interestingly, it also led to a complete loss of intracellular S100a8 (Figure 1B). While S100a8 has been shown to have functions independent of S100a9 (and vice versa), in T lymphocytes it appears that they are dependent upon one another for stabilization and function. Furthermore, we found that T lymphocytes excrete calprotectin, RSDS potentiates production from these cells, and S100a9$^{-/-}$ mice do not produce any detectable secreted calprotectin (Figure 1C). To assess whether T lymphocytes were the primary source of elevated circulating calprotectin after RSDS, we evaluated levels of this protein in recombination activating gene 2 knockout (Rag2$^{-/-}$) mice, which lack mature lymphocytes. Circulating levels of calprotectin in Rag2$^{-/-}$ mice were virtually identical to those in WT mice (Figure S1B), suggesting that the robust increase in T lymphocyte produced calprotectin after RSDS does not contribute significantly to the elevations in circulation.

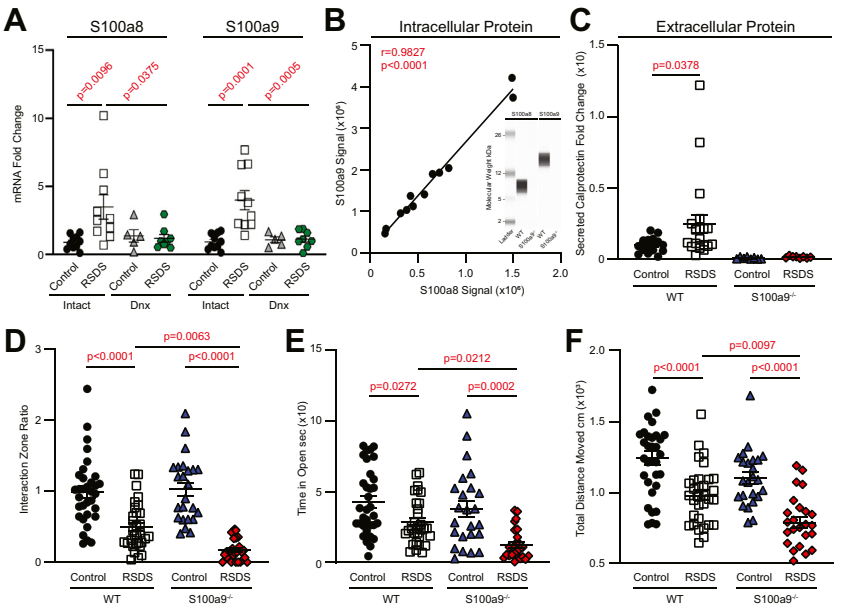

Figure 1. Loss of S100a9 potentiates RSDS-induced behavioral changes. WT and S100a9$^{-/-}$ mice were run through RSDS and behavior testing, after which splenic T lymphocytes were isolated from these animals. **(A)** S100a9 and S100a8 mRNA levels assessed by real-time quantitative polymerase chain reaction in freshly isolated pan T lymphocytes from intact and splenic Dnx mice (n = 9 intact control, 10 intact RSDS, 5 Dnx control, 8 Dnx RSDS). **(B)** S100a8 and S100a9 intracellular protein quantification by Jess automated Western blotting analysis on naïve splenic T lymphocytes (n = 6 control, 6 RSDS). Inset, generated image of S100a8 and S100a9 intracellular protein in WT and S100a9$^{-/-}$ T lymphocytes. **(C)** Extracellular calprotectin in media of cultured T lymphocytes assessed by enzyme-linked immunosorbent assay (n = 6 WT control, 9 WT RSDS, 8 S100a9$^{-/-}$ control, 8 S100a9$^{-/-}$ RSDS). **(D)** Quantification of the interaction ratio from social interaction testing. **(E, F)** Quantification of the time spent in open arms and total distanced moved from elevated zero maze testing (**D**–**F**: n = 33 WT control, 32 WT RSDS, 24 S100a9$^{-/-}$ control, 25 S100a9$^{-/-}$ RSDS). Statistics used include Mann-Whitney U test, Pearson correlation, or two-way analysis of variance with Tukey's post hoc analysis where appropriate. Dnx, denervated; mRNA, messenger RNA; RSDS, repeated social defeat stress; WT, wild-type.

Finally, to extend these observations to include both sexes, we used a juvenile model of RSDS in which prepubescent mice are exposed to the 10-day RSDS protocol with an aggressive adult male CD1 and then singly housed for 4 weeks prior to behavioral and physiological analysis. In this model, both male and female mice displayed significantly elevated S100a8 and S100a9 mRNA in splenic T lymphocytes 1 month after RSDS (Figure S1C). Taken together, these data provide evidence for the first time that T lymphocytes generate S100a8 and S100a9 protein and that expression of calprotectin is elevated both acutely and chronically following RSDS.

Deletion and Inhibition of S100a9 Significantly Worsens the RSDS Behavioral Phenotype

To understand whether elevated calprotectin played a functional role after psychological trauma, we used the S100a9$^{-/-}$ mouse to examine the consequences of calprotectin loss. As expected, RSDS mice showed decreased prosocial behavior, with increased anxiety-like behavior in WT animals (Figure 1D–F). However, loss of S100a9 showed a worsened phenotype with less variability in these behavioral parameters compared with WT mice (Figure 1D–F). To understand whether elevated circulating calprotectin played a functional role after psychological trauma, we pharmacologically inhibited calprotectin by the use of paquinimod. Paquinimod is a clinically used inhibitor that prevents S100a9 from binding to RAGE (receptor for advanced glycation end products) and TLR4 (toll-like receptor 4) receptors. Paquinimod infusion significantly worsened the social interaction ratio after RSDS compared with vehicle-infused animals but did not have a significant impact on anxiety-like behavior between the 2 groups (Figure S2A–C). Together, these data highlight the complexity of the effects of calprotectin on behavior in that total loss of the protein affects both social avoidance and anxiety-like behavior, while extracellular antagonism seems to affect only social avoidance.

Loss of S100a9 Exacerbates RSDS-Induced Inflammation

We previously identified a specific subset of circulating inflammatory proteins (i.e., IL-2, IL-6, IL-17A, IL-22, and tumor necrosis factor α) that are induced after RSDS (19,30–32). Given that S100a9 has numerous reported proinflammatory properties, we originally speculated that this inflammatory phenotype of RSDS would be attenuated in S100a9$^{-/-}$ mice. Counter to this hypothesis, we observed that S100a9$^{-/-}$ RSDS mice showed the same or a significantly exacerbated inflammatory profile as compared with WT RSDS mice, whether it be in circulation, freshly isolated T-lymphocyte mRNA levels, or cytokines produced from T lymphocytes artificially activated ex vivo for 72 hours (Figure 2A–C). The broad composition of immune cells was not altered with the loss of S100a9 (Figure S3A, B), suggesting that the function of immune cells (as opposed to loss/gain) is affected due to the lack of calprotectin. Moreover, exogenous supplementation of calprotectin on unstressed cultured T lymphocytes attenuated both IL-6 and IL-17A mRNA expression compared with untreated control animals (Figure S4A, B). In summary, we found that the loss of S100a9 intensifies inflammation after RSDS and may in fact play a protective or anti-inflammatory role in T lymphocytes.

Loss of S100a9 Alters Mitochondrial Redox and Metabolism in T Lymphocytes

Our laboratory has previously shown that mitochondrial superoxide and metabolism regulates T-lymphocyte inflammation and that T-lymphocyte mitochondrial superoxide is elevated following psychological trauma (19,33,34). Herein, we confirmed this phenomenon in WT animals but found that the loss of S100a9 further potentiated mitochondrial superoxide in T lymphocytes after RSDS (Figure 3A). Similar to S100a8 and S100a9 mRNA expression, this phenomenon was confined only to spleen and blood T lymphocytes, but not T lymphocytes in peripheral lymph nodes (Figure S5A). Paquinimod infusion mirrored this potentiation of T-lymphocyte mitochondrial superoxide (Figure S5B). Moreover, exogenous supplementation of calprotectin in culture decreased mitochondrial superoxide levels in both CD4$^+$ and CD8$^+$ T lymphocytes compared with untreated control animals (Figure S5C). Together, these data suggest that calprotectin plays a previously unidentified role in regulating the mitochondrial redox environment in T lymphocytes.

Understanding that the mitochondrial redox environment is tightly coupled to metabolism (35), we next examined metabolism in the context of RSDS. RSDS significantly enhanced the basal respiration, spare respiratory capacity, and maximum respiration in pan T lymphocytes from WT animals (Figure 3B–E), suggesting enhanced mitochondrial metabolism in these cells after psychological trauma. Interestingly, the loss of S100a9 seemed to increase these same parameters in T lymphocytes at baseline, with RSDS having little to no effect on these cells regarding these metabolic parameters (Figure 3B–E). Examining the metabolic state further, we found that loss of S100a9 was associated with a significant enhancement of both glycolytic and mitochondrial metabolism in purified CD8$^+$ T lymphocytes (Figure 3F–O), with only modest effects in purified CD4$^+$ T lymphocytes (Figure S6A–J). These metabolic alterations with the loss of S100a9 cannot be explained by a difference in mitochondrial mass or proliferative capacity because these were unchanged between WT and S100a9$^{-/-}$ T lymphocytes (Figure S6K–M). Together, these data support that calprotectin plays a major metabolic homeostatic role in T lymphocytes.

Loss of S100a9 Alone Mimics RSDS-like Gene Expression Patterns in T Lymphocytes

Due to S100a9 significantly affecting mitochondrial superoxide levels, inflammation, and metabolism in T lymphocytes from RSDS mice, we performed single-cell RNA sequencing analysis on splenocytes from WT and S100a9$^{-/-}$ control and RSDS mice (3 mice in each group, 12 mice total) to obtain a greater understanding of the gene expression changes in T lymphocytes due to these perturbations. After compiling the data using the uniform manifold approximation and projection analysis (Figure 4A), we identified cell type clusters using the Tabula Muris (36). Examining the differential gene

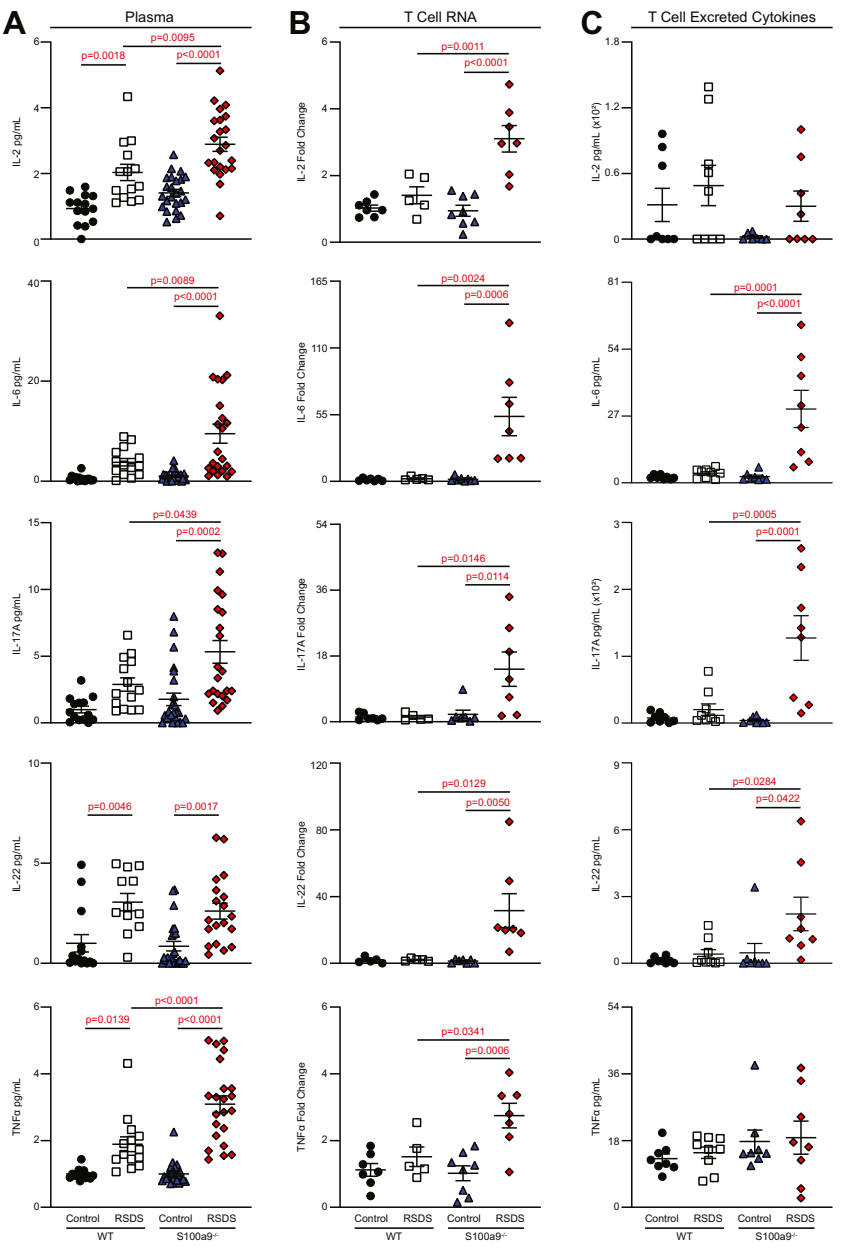

Figure 2. Loss of S100a9 exacerbates the inflammatory phenotype displayed after RSDS. WT and S100a9$^{-/-}$ mice were run through RSDS, after which plasma and splenic T lymphocytes were isolated from these animals. **(A)** Circulating cytokines in plasma assessed by Mesoscale Discovery (n = 14 WT control, 14 WT RSDS, 24 S100a9$^{-/-}$ control, 22 S100a9$^{-/-}$ RSDS). **(B)** T-lymphocyte inflammatory cytokine messenger RNA assessed by quantitative real-time polymerase chain reaction (n = 7 WT control, 5 WT RSDS, 8 S100a9$^{-/-}$ control, 7 S100a9$^{-/-}$ RSDS). **(C)** Excreted inflammatory cytokines in media of cultured T lymphocytes assessed by Mesoscale Discovery (n = 8 WT control, 9 WT RSDS, 8 S100a9$^{-/-}$ control, 8 S100a9$^{-/-}$ RSDS). Statistics used include two-way analysis of variance with Tukey's post hoc analysis throughout. IL, interleukin; RSDS, repeated social defeat stress; TNF, tumor necrosis factor; WT, wild-type.

expression of the CD4$^+$ and CD8$^+$ T-lymphocyte clusters, we found that RSDS induced a robust alteration in the genetic signature of both WT and S100a9$^{-/-}$ T lymphocytes (Figure 4B, C). Intriguingly, pathway analysis identified identical top canonical pathways altered in both WT and S100a9$^{-/-}$ T lymphocytes after RSDS (Figure 4C), with translation regulation and mitochondrial function being disrupted in both genotypes. Furthermore, no differences were noted between CD4$^+$ and CD8$^+$ T lymphocytes, suggesting that the changes associated with RSDS or S100a9$^{-/-}$ loss have a universal effect on T lymphocytes. In the absence of RSDS, the loss of S100a9 alone seemed to significantly impact translation regulation and metabolic signaling (Figure 4C), thus suggesting that S100a9$^{-/-}$ T lymphocytes possess a phenotype at baseline similar to that of WT T lymphocytes from an animal exposed to RSDS. This may explain why S100a8 and S100a9 were the top upregulated genes in our previous analysis of WT RSDS T lymphocytes (19), as they may act as critical regulators of the processes disrupted by RSDS. Taken together, RSDS significantly affects anabolic and metabolic genetic pathways in T lymphocytes, while the loss of S100a9$^{-/-}$ shifts T lymphocytes into an RSDS-like state even in the absence of psychological trauma.

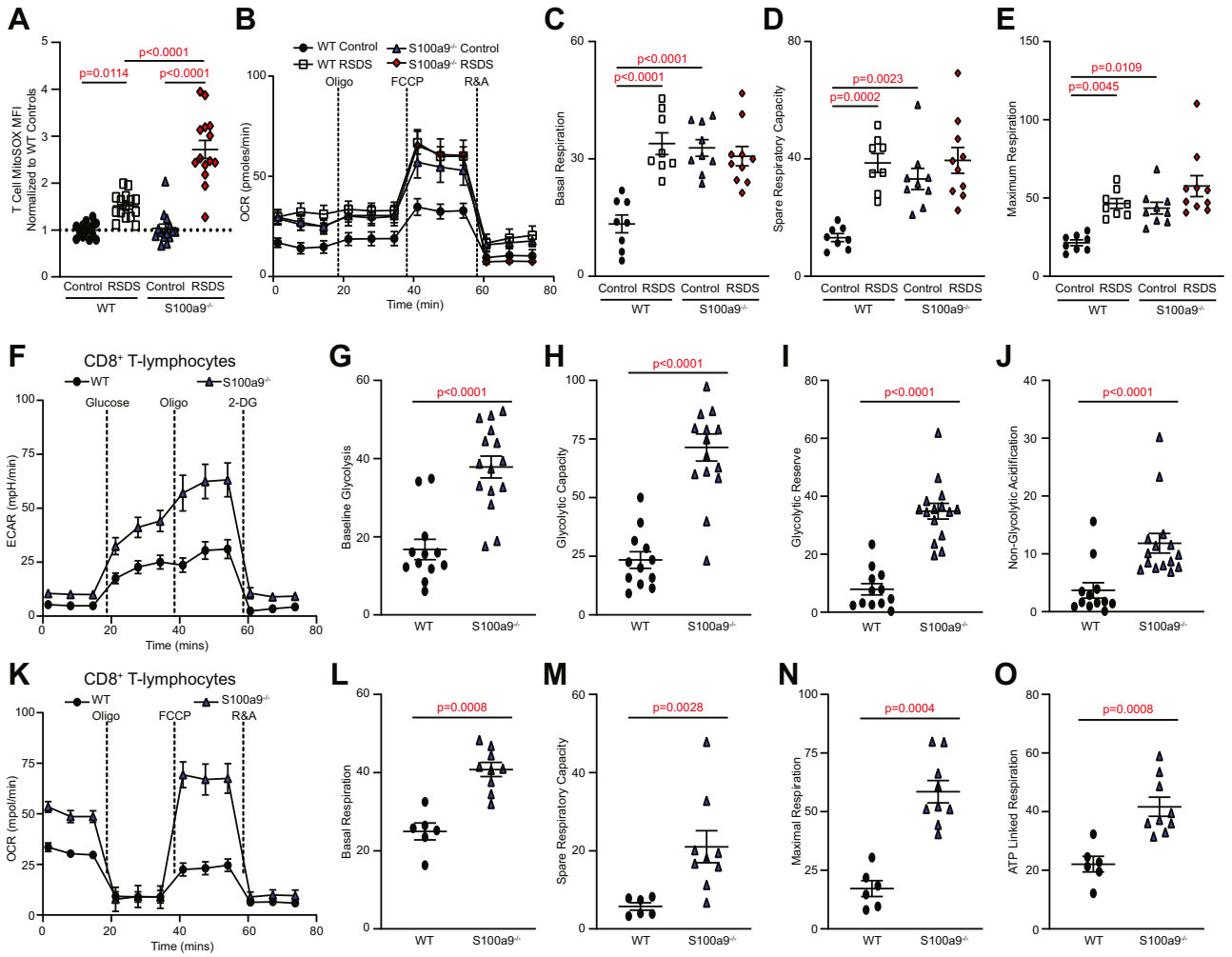

Figure 3. Loss of S100a9 disrupts T-lymphocyte mitochondrial and metabolic homeostasis. WT and S100a9$^{-/-}$ mice were run through RSDS, after which splenic T lymphocytes were isolated from these animals. **(A)** MitoSOX Red MFI assessed by flow cytometry. Data normalized WT control T lymphocytes to control for interexperimental variance (n = 14 WT control, 15 WT RSDS, 14 S100a9$^{-/-}$ control, 14 S100a9$^{-/-}$ RSDS). **(B)** Representative OCR curve of mitochondrial stress test (n = 6 WT control, 5 WT RSDS, 9 S100a9$^{-/-}$ control, 10 S100a9$^{-/-}$ RSDS). **(C–E)** Quantification of various metabolic states from the mitochondrial stress test. **(F)** Representative ECAR curve of a glycolysis stress test (n = 12 WT, 15 S100a9$^{-/-}$). **(G–J)** Quantification of metabolic states from the glycolytic stress test. **(K)** Representative OCR curve of mitochondrial stress test (n = 6 WT, 9 S100a9$^{-/-}$). **(L–O)** Quantification of metabolic states from the mitochondrial stress test. Statistics used include Mann-Whitney U test or two-way analysis of variance with Tukey's post hoc analysis where appropriate. ECAR, extracellular acidification rate; FCCP, carbonyl cyanide p-trifluoro methoxyphenylhydrazone; MFI, mean fluorescent intensity; OCR, oxygen consumption rate; R&A, rotenone and antimycin; RSDS, repeated social defeat stress; WT, wild-type.

DISCUSSION

Calprotectin has been reported to act primarily as a proinflammatory protein, and its involvement in inflammatory diseases such as cancer, rheumatoid arthritis, psoriasis, endotoxin-induced shock, and obesity are well defined (20,21,23,37–44). Furthermore, calprotectin is being investigated as a potential clinical biomarker for several inflammatory bowel, periodontal, autoimmune, and infectious diseases (including COVID-19) because of how strongly correlated the protein is with inflammatory profiles in these disease states (45–48). Additional evidence of the proinflammatory role of calprotectin is supported by the pharmacological inhibition of the protein using paquinimod, which demonstrates anti-inflammatory and beneficial effects in several of the aforementioned inflammatory pathologies (49–54). However, examinations of calprotectin in the context of mental health are scarce.

To our knowledge, only 3 reports exist that have identified S100a8 and S100a9 upregulation in models of psychological stress, and all 3 reported that the upregulation occurred only in regions of the brain (55–57). Of these 3 studies, only 1 attempted to examine the mechanistic role of calprotectin (56). In that work, Gong *et al.* (56) examined the phenotypic effects of centrally administered recombinant calprotectin or paquinimod in a mouse depression model of chronic unpredictable stress. Consistent with our original hypothesis, they observed that centrally infused calprotectin exacerbated depressive-like

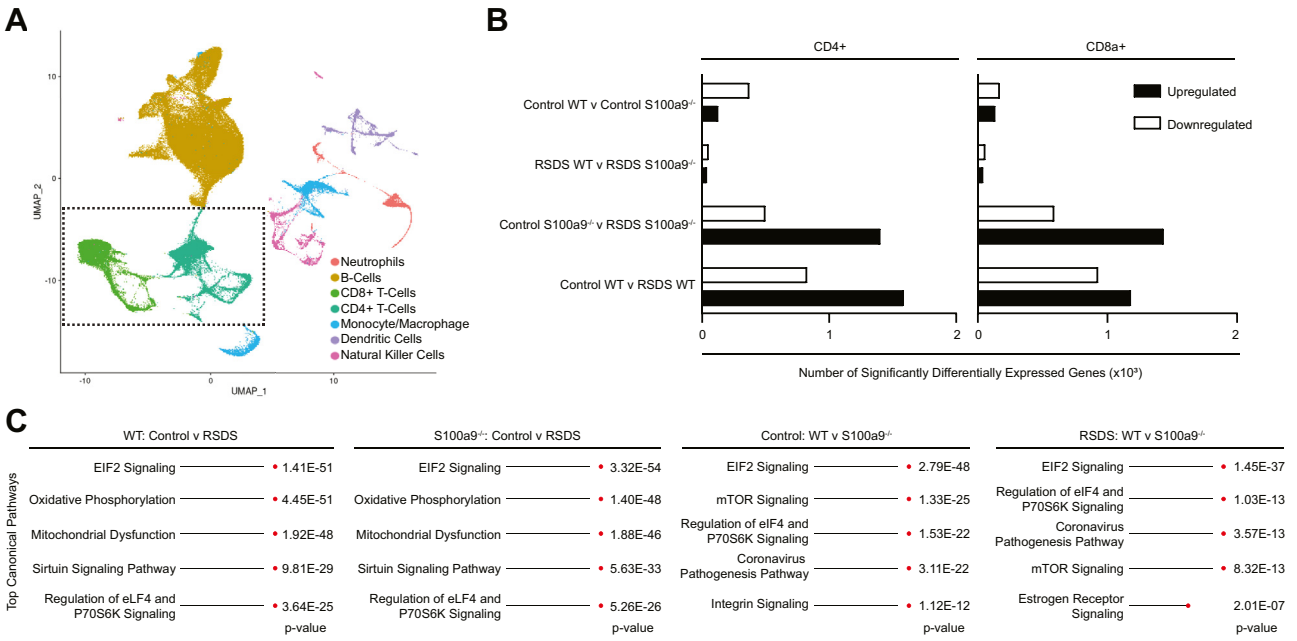

Figure 4. Loss of S100a9 perturbs T-lymphocyte gene expression similar to RSDS. WT and S100a9$^{-/-}$ mice were run through RSDS, after which total splenocytes were isolated from these animals and assessed by single-cell RNA sequencing. **(A)** Seven primary-cell population clusters were identified within the UMAP. **(B)** Quantification of number of significant differentially regulated genes among comparisons of genotype and psychological trauma. **(C)** Ingenuity Pathway Analysis of differentially regulated genes among comparisons of genotype and psychological trauma in CD8^{+} and CD4^{+} T lymphocytes (not shown because identical to CD8^{+}). mTOR, mechanistic target of rapamycin; RSDS, repeated social defeat stress; UMAP, uniform manifold approximation and projection; WT, wild-type.

behavior and neuroinflammation, while pharmacological inhibition of calprotectin was shown to be beneficial in their animal model. Moreover, paquinimod was shown to directly attenuate reactive oxygen species production from a cultured microglial cell line, which further supported an anti-inflammatory effect of calprotectin inhibition (56). This singular study supports the notion that central calprotectin plays a proinflammatory and propsychopathological role during depression; however, our findings suggest differential effects in RSDS.

The disparity in these results may be due to several factors. First, we used different models of stress induction, which have different timelines, stressors, and phenotypes. The differences in our findings may demonstrate the complexity of calprotectin expression among psychopathologies, and a one-size-fits-all approach may not be accurate. Second, our primary focus was on the effects of systemic loss of calprotectin, while the others examined the consequences specifically in the brain. It is quite possible that calprotectin plays differential roles that are cell type dependent, and targeted manipulation may demonstrate differential effects to global loss. While this may complicate therapeutic approaches with calprotectin, it does not minimize the possible role that this protein may play as a biomarker of psychopathologies and their progression. Finally, the dosage and route of administration of paquinimod differ between the studies. We used constant infusion by way of osmotic minipumps to block calprotectin, while the previous report administered paquinimod by intraperitoneal injection. These methods of administration are significantly different in that 1) no handling or unwanted stress is induced with osmotic minipumps, and 2) the constant infusion by osmotic minipumps limits the bolus and taper effects of intraperitoneal injections. Furthermore, the dose we chose was in the lower range reported in the literature to minimize off-target effects (53). Therefore, while the findings from these studies may seem contrasting, they may in fact highlight important nuances of calprotectin expression, function, dosage, and timing during psychological stress.

The question still remains as to how the loss of calprotectin worsens RSDS-induced behavior. While it may be possible that the proinflammatory T-lymphocyte changes we observed may contribute to the potentiated behavioral changes, we cannot make that conclusion herein given the correlative nature of the data at this time. In addition to T lymphocytes, microglia and monocytes have been shown to play a significant role in behavior modification in RSDS (58,59), and these cells also express and respond to calprotectin. It may be possible that the behavior effects observed herein may be due to changes in innate as opposed to adaptive immune cells. Another possibility is that calprotectin plays a regulatory role in neurons similar to that in T lymphocytes. Given that calprotectin possesses calcium and redox modulatory characteristics, the loss of this protein in neurons may lead to unrestricted signaling by these moieties. This could lead to unregulated and accelerated neuronal firing, thus potentiating behavioral changes after RSDS. As mentioned above, as part of large-scale genetic screens, calprotectin has previously been reported to be upregulated in behavior-regulatory regions of the brain such as the hippocampus and amygdala after

psychological stress (55,57). These findings would support the notion that calprotectin is upregulated in response to stress as a form of negative feedback regulation, but additional studies using cell type–specific knockouts of calprotectin are needed to address the question of which specific cells are producing calprotectin in the brain as well as the intracellular versus extracellular effects of this protein on behavior.

In addition to the brain, it still remains unclear what specific signals increase calprotectin expression in T lymphocytes during RSDS. Herein, we show differential effects on T lymphocytes depending upon where they are located in the body (i.e., spleen, blood, and lymph nodes). While spleen and blood T lymphocytes appeared to increase calprotectin and mitochondrial oxidation, no effect was observed in the inguinal or mesenteric lymph node T lymphocytes, which suggests the potential for regional or organ-specific responses to psychological trauma. It is possible that T lymphocytes located in the central nervous system may also be differentially affected and may play a greater role in the behavioral phenotypes of RSDS as suggested by previous work that has examined these adaptive immune cells in the brain (60). Moreover, we showed that targeted denervation of the autonomic nerve to the spleen completely reverses T-lymphocyte upregulation of calprotectin after RSDS. This finding demonstrates two things. First, it shows that circulating factors that are elevated during RSDS (e.g., corticosterone) are not contributing to the increase in T-lymphocyte calprotectin because these would still remain elevated in the absence of splenic denervation. Second, understanding that the splenic nerve is exclusively catecholaminergic (61), this suggests that the primary upstream signal leading to the upregulation of calprotectin in T lymphocytes is likely sympathetic derived. While controversy still remains regarding parasympathetic versus sympathetic input into the splenic nerve (62–64), the output of the splenic nerve comprises catecholamines and other sympathetic neuropeptides (e.g., substance P, neuropeptide Y). While it is possible that these neurotransmitters are acting directly on T lymphocytes, it is just as likely that they are acting on other immune cells first, which then secondarily communicate with T lymphocytes. Studies have clearly demonstrated sympathetic-mediated effects of psychological stress on innate immune cells (58,59,65–69), which could in turn produce an exhaustive list of secondary messengers that may affect T-lymphocyte physiology. Work is underway in our laboratory examining these hypotheses to better understand the specific molecular pathway from psychological trauma to T-lymphocyte inflammation.

Calprotectin is commonly referred to as a proinflammatory protein and a damage-associated molecular pattern due to its reported ability to activate RAGE and TLR4 receptors (20). While our work presented herein challenges this canonical proinflammatory characteristic of calprotectin, others have also found that S100a8 and S100a9 provide protective functions in various contexts. For example, S100a8 administration was shown to induce anti-inflammatory IL-10 and protect against acute lung injury (70). In another study, loss of S100a9 potentiated the development of autoimmunity in a mouse model of lupus, suggesting that S100a9 played a protective role in this context (71). Another report found that S100a9-deficient neutrophils, macrophages, and dendritic cells all demonstrated differential cytokine expression in a model of atherosclerosis (72). Because not all cell types showed a similar pattern of inflammation, Averill *et al*. (as well as others) concluded that the posttranslational, cellular, and microenvironmental contexts must play a significant role in how S100a8 and S100a9 function regarding inflammation (72,73). Given that our work presented herein is the first examination of calprotectin in T lymphocytes, it seems that in the context of psychological trauma these proteins play a protective and anti-inflammatory role.

One interesting observation from this work is that paquinimod was not able to fully recapitulate the complete phenotype produced by total knockout of S100a9. We believe that this is because paquinimod is only able to antagonize calprotectin binding to RAGE or TLR4 receptors but would have no impact on other receptors or intracellular functions of calprotectin. For example, we observed that paquinimod potentiated social avoidance but had no effect on anxiety-like behavior. The fact that paquinimod affected social avoidance suggests that this behavior is susceptible to calprotectin binding to RAGE or TLR4. In contrast, only S100a9 knockout affected anxiety-like behavior, which suggests a potential role for intracellular calprotectin in that neural pathway. Similarly, we observed that paquinimod could mimic the potentiation of T-lymphocyte mitochondrial superoxide but did not affect inflammatory gene or protein expression similar to loss of S100a9 (data not shown). Again, these data uncover a duality of extracellular and intracellular functions of calprotectin on T lymphocytes that have not yet been reported.

While we previously demonstrated calprotectin gene expression in T lymphocytes (19), the work presented herein reports the first confirmed functional roles for the protein in these adaptive immune cells. First, it seems that S100a9 (and S100a8 because this protein is also lost with the knockout of S100a9) plays a significant role in the maintenance of metabolic homeostasis. As we observed in the absence of psychological trauma, both mitochondrial and glycolytic metabolism are greatly enhanced in T lymphocytes isolated from S100a9$^{-/-}$ mice. How calprotectin regulates T-lymphocyte metabolism remains unknown, but we hypothesize that the mechanism may involve intracellular calcium sequestration. It is well established that calcium signaling plays an essential role in T-lymphocyte activation, proliferation, differentiation, and metabolism (74–76). Additionally, mitochondrial calcium uptake enhances mitochondrial energy output (77–80), which has been shown to be essential for T-lymphocyte polarization shifts from naïve, activated, and memory states (35,81–83). With this, calcium must be tightly regulated and controlled to avoid aberrant activation or differentiation, and calprotectin may act as an intracellular calcium sequestration protein to serve this purpose. Calprotectin is able to bind 6 calcium ions (84), so it may act as a buffer to inhibit excess intracellular calcium signaling during T-lymphocyte activation. This hypothesis is supported by our data demonstrating that loss of calprotectin produced a hyperactivated T-lymphocyte state with pronounced metabolism, inflammation, and redox consequences.

Our original hypothesis that calprotectin may be perpetuating the negative inflammatory and redox consequences of

RSDS in T lymphocytes stemmed from our original observation that T lymphocyte–expressed calprotectin was highly correlated with mitochondrial superoxide levels after RSDS (19). Given the previously discussed proinflammatory descriptions of calprotectin, it was logical to assume that this protein was playing a similar role in the RSDS context. However, our results show quite the opposite and suggest that calprotectin may be playing a protective role in these cells. This suggests that calprotectin expression may occur in response to the altered redox or inflammatory environments as opposed to being the cause. This concept is supported by early work in our laboratory examining the consequences of enhanced mitochondrial superoxide in T lymphocytes. Reviewing this previous work in which we used a mouse model of T lymphocyte–specific manganese superoxide dismutase knockout (to create an animal with uncontrolled mitochondrial superoxide in T lymphocytes), we found that S100a8 and S100a9 were indeed elevated as assessed by an Affymetrix gene array (85). These data suggest that calprotectin upregulation in T lymphocytes is downstream to mitochondrial redox changes and may serve as a protective checkpoint necessary to prevent excessive activation or inflammation from T lymphocytes. Future studies will examine whether normalization of the mitochondrial redox environment in T lymphocytes following RSDS is sufficient and/or necessary to restore normal calprotectin expression and inflammation in these cells.

Our data demonstrate a strong mitochondrial reaction in T lymphocytes after RSDS, which has also been observed recently in the brains of chronically stressed animals and humans. Work from Carmen Sandi's group (86) has shown that psychological stress significantly affects mitochondrial gene signatures in the prefrontal cortex of both mice and humans. However, in that work, they demonstrated that mitochondrially encoded components of the electron transport chain are significantly elevated in the brain after stress, while we demonstrate the opposite in T lymphocytes. Furthermore, their observed mitochondrial gene changes in the brain lead to a decreased metabolic state, whereas our observed changes seem to enhance mitochondrial respiration. These disparities are likely a consequence of the differential metabolic demands that the individual cell types require during psychological stress. For example, T lymphocytes often proliferate during times of activation, which requires an abundance of ATP, whereas neurons do not. Additionally, the vast majority of mitochondrial-related genes in our dataset (94 in total) encode for the 5 major complexes of the electron transport chain. Intriguingly, of these 94 genes, all nuclear-encoded mitochondrial genes were upregulated, while, as previously mentioned, all mitochondrial-encoded mitochondrial genes were downregulated. This dichotomy suggests a compensatory upregulation of nuclear-encoded transcripts to counterbalance this defect. This exact phenomenon is observed with the loss of S100a9 in T lymphocytes even in the absence of psychological trauma, which demonstrates the previously undescribed importance of this protein in the maintenance of T-lymphocyte mitochondrial homeostasis. These similar genetic patterns are likely not random, but rather explain why S100a8 and S100a9 are two of the most upregulated genes in T lymphocytes after RSDS. It is unclear whether S100a8 or S100a9 were altered in the aforementioned work from Carmen Sandi, but it would be interesting to examine this in the context of other examples of chronic psychological stress. Overall, our data suggest that calprotectin regulates the T-lymphocyte processes altered by psychological trauma, and thus, the loss of calprotectin alone leads to the net effect of increased cellular metabolism, enhanced mitochondrial superoxide production, and elevated inflammation similar to that of RSDS.

While these data provide new insight into psychological trauma–induced inflammation, this study is not without limitations. First, the mice used for these studies were constitutive S100a9 knockouts, which limits our ability to make cell type–specific conclusions regarding systemic processes (e.g., behavior or circulating inflammation). At the time of this work, no conditional S100a9 knockout mouse had been developed. This does not diminish the findings of this study but encourages the development of a conditional S100a9 knock-out animal for use in more nuanced studies. Second, we have not performed a calprotectin rescue experiment in S100a9$^{-/-}$ mice. We did attempt these studies using osmotic minipumps similar to that of paquinimod but were unable to verify calprotectin in circulation of S100a9$^{-/-}$ mice. At this time, we are unsure whether this is due to a rapid breakdown of this protein when infused or an unknown technical limitation. Furthermore, the infusion of extracellular calprotectin will not restore intracellular protein in T lymphocytes. This is additional incentive to develop a conditional S100a9$^{-/-}$ mouse model with which we can truly address the role of intracellular versus extracellular calprotectin on T lymphocytes. Finally, most studies herein were performed on male mice. This is due the inability of female mice to be incorporated into the standardized RSDS paradigm. We have successfully incorporated females using an adapted version with juvenile experimental mice, but using mice at this young age poses numerous other challenges. We are currently adapting other established methods of female RSDS into our laboratory (87,88) and hope to follow up these studies with S100a9$^{-/-}$ female mice.

In summary, we put forth data supporting a protective role for calprotectin in T lymphocytes after psychological trauma. While "protection" is in the protein's namesake, this term was likely given to calprotectin due to its canonical ability to sequester calcium and metals, which protects from bacterial and other pathogen infections. These established roles for calprotectin primarily come from studies with neutrophils, where calprotectin makes up approximately 60% of neutrophil cytoplasmic protein, is rapidly excreted during times of infection, and is expressed at over 3 orders of magnitude the levels expressed in T lymphocytes (which we suppose may be the reason that it has not been described in T lymphocytes to date). Our findings pave a new road for this protein in the context of T lymphocytes and other cells where the role for calprotectin remains undefined.

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ARTICLE INFORMATION

From the Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, Nebraska (CMM, SKE, GFW); Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska (GFW); Department of Psychiatry and Behavioral Sciences, Texas A&M University, Bryan, Texas (JD, ZNT, ECR, THL, AJCl, VIV, AJCa); Department of Medical Physiology, Texas A&M University, Bryan, Texas (ECR, THL, AJCl, AJCa); Department of Psychiatry, University of Arizona, Phoenix, Arizona (VIV); and Lieber Institute for Brain Development, Johns Hopkins University, Baltimore, Maryland (VIV).

Address correspondence to Adam J. Case, Ph.D., at acase@tamu.edu.

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