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RKIP Contributes to IFN-γ Synthesis by CD8$^+$ T Cells after Serial TCR Triggering in Systemic Inflammatory Response Syndrome

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Systemic inflammatory response syndrome (SIRS) is associated with the development of severe medical complications, including progression to multiple organ dysfunction syndrome and even death. To date, only marginal improvements in terms of therapeutic options have been established for patients affected by SIRS. Raf kinase inhibitor protein (RKIP) is a regulator of MAPK and NF-κB signaling cascades, which are both critical for production of the proinflammatory cytokines responsible for SIRS initiation. By testing a T cell–dependent mouse model of SIRS that utilizes staphylococcal enterotoxin A specific for Vβ3$^+$ T cells, we show that RKIP is necessary for the exaggerated production of IFN-γ from SIRS splenocytes. This effect was not due to differences in T cell expansion, IL-10 production, or APC priming, but rather a cell-intrinsic defect lying downstream of the TCR in staphylococcal enterotoxin A–specific CD8$^+$ T cells. Importantly, mice lacking RKIP were still able to proliferate, survive, and contribute to cytokine production in response to pathogen associated molecular pattern-TLR–mediated stimuli, despite the TCR-dependent defects seen in our SIRS model. Finally, by blocking RKIP in wild-type SIRS splenocytes, the IFN-γ response by CD8$^+$ Vβ3$^+$ T cells was significantly diminished. These data suggest that RKIP may be a potential therapeutic target in SIRS by curbing effector cytokine production from CD8$^+$ T cells during serial TCR triggering. ***The Journal of Immunology*****, 2013, 191: 708–716.**

Systemic inflammatory response syndrome (SIRS) results from the general release of large quantities of proinflammatory cytokines into circulation. This cytokine storm has the potential to lead to many clinical complications for patients, including respiratory failure from acute respiratory distress syndrome, gastrointestinal bleeding, anemia, deep vein thrombosis, metabolic abnormalities, hypotension, disseminated intravascular coagulopathy, multiple organ dysfunction syndrome, and many times death (1, 2). SIRS can be prompted from many initiators, including infectious and noninfectious etiologies. These triggers range from uncontrolled bacterial, viral, and fungal infections to pathogenic toxin exposure, organ ischemia, trauma, autoimmune disorders, pancreatitis, hemorrhage, and substance abuse. Several studies have shown that between 30 and 60% of all hospital admissions meet the clinical diagnostic criteria for SIRS (3, 4). Even though not all patients who meet these criteria progress to severe sequelae, SIRS remarkably carries a baseline mortality rate of ∼7%, which climbs to >40% if the patient develops symptoms of shock (3). Taken together, it is no surprise that SIRS is both a widespread and costly problem for health care systems nationally and globally (5).

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Abbreviations used in this article: CARS, compensatory anti-inflammatory response syndrome; CTM, complete tumor medium; LDH, lactate dehydrogenase; PAMP, pathogen associated molecular pattern; RKIP, Raf kinase inhibitor protein; SEA, staphylococcal enterotoxin A; SIRS, systemic inflammatory response syndrome; Treg, T regulatory cell; TSST-1, toxic shock syndrome toxin-1.

Despite affecting a large number of patients, few therapeutics exist for SIRS. Clinical trials attempting to inhibit inflammatory factors such as TNF-α and IL-1β failed to show significant efficacy (6–9). A current therapeutic regimen typically involves an antimicrobial agent, if an infection is present; medications to restore cardiac and respiratory abnormalities if needed; and a broadly immunosuppressive corticosteroid (10, 11). Using drugs that inhibit beneficial inflammatory responses in patients who have either concomitant infections or increased susceptibility due to hospitalization is likely to be counterproductive.

SIRS is very difficult to study in humans because the onset and progression are rapid, and it is most likely challenging to enroll patients who are acutely ill into clinical studies. Also, because of its heterogeneity of origins, no unified mouse model of SIRS exists. We sought to use a model system that was clinically relevant to human disease, which contained a known trigger of human SIRS that followed the natural history of the disease in terms of its acute onset and patterns of systemic cytokine release. One model incorporating these important facets of human SIRS is exposure to staphylococcal enterotoxin A (SEA). SEA is produced by the human pathogen *Staphylococcus aureus*, and other *S. aureus* enterotoxins, such as toxic shock syndrome toxin-1 (TSST-1), induce rapid release of proinflammatory cytokines into the systemic circulation in significant quantities and, importantly, can cause SIRS in humans (12–14). This robust cytokine storm is mediated by the rapid expansion and activation of T cells that specifically bear the Vβ3 chain of the TCR (15). In addition, exposure to these superantigens has explicitly illustrated many other aspects of SIRS pathology, including the induction of acute lung injury after vascular damage (16–18) as well as transient immunosuppression similar to the compensatory anti-inflammatory response syndrome (CARS) seen in a number of SIRS patients (2, 19, 20).

The major cytokine network in SIRS involves production of proinflammatory factors, such as IL-6, IFN-γ, and TNF-α, which are dependent on the NF-κB and the MAPK signaling pathways

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(21, 22). It is critical to discover ways to modulate these cascades to control SIRS without affecting immunocompetence. Raf kinase inhibitor protein (RKIP) negatively regulates these pathways by binding and inhibiting the kinase activities of several important signaling factors, including Raf, MEK, ERK, TRAF6, TAK1, NIK, and IKKα/β (23–26). RKIP has also been associated with metastatic disease in many human cancers, including prostate (27) and breast (28), but its role in the immune system is undefined.

In this study, we report that serial triggering of the TCR with SEA models many aspects of human SIRS and identifies IFN-γ as a potential intersection between the damage associated with SIRS and the diminished inflammation seen in CARS. This is illustrated by the fact that wild-type T cells continue to make IFN-γ in SIRS even if they fail to make IL-2, and thus retain the capability to potentiate disease. Importantly, RKIP is shown to be a critical player in these processes because genetic loss of this protein prevents the ability of specific T cells to make exorbitant amounts of IFN-γ while only moderately affecting the anti-inflammatory cytokine, IL-10. In addition, by inhibiting RKIP using the small molecule inhibitor locostatin (29), IFN-γ production was blocked in wild-type SIRS T cells. The data in this work suggest that RKIP may be a key therapeutic target for dampening the robust inflammation seen in SIRS while preserving the CARS response.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). $RKIP^{-/-}$ mice were engineered by the Dr. Jan Kylsik Laboratory (Department of Neuroscience, Brown University, Providence, RI) (30) and received from the laboratory of Dr. Kam Yeung (Department of Biochemistry and Cancer Biology, University of Toledo, Toledo, OH). Once in house, $RKIP^{-/-}$ mice were rederived by the Gene Targeting and Transgenic Facility at the University of Connecticut Health Center following a standard protocol (31). After successful rederivation, $RKIP^{-/-}$ were bred to C57BL/6J mice to obtain wild-type littermate controls. All mice at University of Connecticut Health Center were housed in specific pathogen-free conditions and handled in accordance with institutional and federal guidelines outlined by the National Institutes of Health.

Immunizations

For SIRS induction, staphylococcal enterotoxin A (SEA; Toxin Tech, Sarasota, FL) was injected at 1 μg per mouse diluted in 0.2 ml balanced salt solution i.p. Forty-eight hours later, a second i.p. injection of SEA was administered and tissues were harvested at 48 or 72 h after the second injection (2°). For pathogen associated molecular pattern (PAMP)-TLR studies, 1 μg SEA was i.p. injected, followed by an i.p. injection of 10 μg LPS derived from *Salmonella typhimurium* (Sigma-Aldrich, St. Louis, MO) 18 h after SEA, and tissues were harvested 12 d after SEA administration.

Tissue processing

Spleens were crushed through 100-μm nylon mesh strainers (Falcon/BD Biosciences, San Jose, CA) and treated with ammonium chloride for 5 min at room temperature to lyse RBCs. Pooled peripheral lymph nodes (inguinal, axillary, and brachial) were crushed through nylon mesh strainers. Liver leukocytes were obtained, as previously described (32). Briefly, livers were perfused using a solution of PBS and sodium heparin (Sigma-Aldrich) crushed through nylon mesh strainers, and separated by a 35% Percoll gradient (Sigma-Aldrich). Blood was obtained from the tail veins of mice before and 1.5 h after 2° SEA administration. Blood was kept at room temperature for 30 min to allow for coagulation and then stored for 1 h at 4°C to shrink the absolute size of the clot. Samples subsequently underwent centrifugation at 13,000 rpm at 4°C for 10 min, and the upper aqueous fraction (serum) was collected and stored at −80°C until analysis.

Cell purification and sorting

For studies involving purified cell populations, splenocytes were isolated, as described, and were subjected to depletion using MicroBeads specific for CD8, CD4, and DX5 (Miltenyi Biotec, Gladbach, Germany) and MACS LD purification columns (Miltenyi Biotec) following the manufacturer's protocol to obtain purified splenic APCs, or subjected to positive selection using CD8 or CD4 MicroBeads and MACS LS purification columns (Miltenyi Biotec) to obtain purified CD8 and CD4 T cell populations.

Cell culturing and flow cytometry

For in vitro restimulations, 5×10^5 cells were cultured at 37°C and 5% CO_2 in 0.2 ml complete tumor medium (CTM), which consists of MEM supplemented with 10% FBS, dextrose, salts, amino acids, and antibiotics. As indicated, cells were restimulated with 0.1 μg/well SEA, 50 ng/ml PMA (Calbiochem, Gibbstown, NJ), plus ionomycin (1 μg/ml) (Invitrogen, Carlsbad, CA). For studies involving locostatin (Calbiochem), 5 μM solutions diluted in CTM were used as the effective dose with an equal volume by percentage of DMSO in CTM acting as the vehicle control. These reagents were used as indicated within the corresponding figure legends. Cultures analyzed for intracellular cytokine production by flow cytometry were stimulated in culture for 4–5 h, whereas cultures being used to assess cytokine production by ELISA were stimulated overnight. The following mAbs were purchased from BD Biosciences: PE-conjugated TNF-α, IL-10, Vβ3; biotin-conjugated CD86; allophycocyanin-conjugated CD44, rat IgG2a, rat IgG2b; FITC-conjugated rat IgG2a, hamster IgG; Alexa Fluor-700–conjugated CD3; PerCP-conjugated CD4, B220, rat IgG2a; and Pacific Blue–conjugated CD8. The following mAbs were purchased from eBioscience (San Diego, CA): PE-conjugated CD11b, CD25, CD80, rat IgG1, rat IgG2b, hamster IgG; biotin-conjugated rat IgG2a; Alexa Fluor 700–conjugated MHC-II, rat IgG2b; allophycocyanin-conjugated IFN-γ, B220, rat IgG1, rat IgG2b; FITC-conjugated Foxp3, CD11c; PE-Cy7–conjugated streptavidin; and PerCP-conjugated rat IgG2a. FITC-conjugated annexin V was purchased from BD Biosciences.

Surface and intracellular staining was performed, as previously outlined (33). Briefly, cells were suspended in a wash buffer containing balanced salt solution, 3% FBS, and 0.1% sodium azide. Blockade of nonspecific binding (Fc block) was performed by treating cells for 10 min prior to initial extracellular Ab staining with a solution containing mouse serum, human IgG, and anti-Fc mAb 2.4G2 (34). Surface staining of 1×10^6 cells/well was performed with the aforementioned Abs at concentrations determined by individual titration studies, ranging from 1:50 to 1:200, for 30 min at 4°C in the dark. After incubation, cells were washed twice with wash buffer to remove any nonbound Ab and were subsequently fixed with 2% paraformaldehyde. For intracellular staining, the cells were additionally permeabilized with wash buffer containing 0.25% saponin (Sigma-Aldrich) and stained with Abs against intracellular Ags overnight at 4°C in the dark. The following day, cells were washed twice to remove any nonbound Ab. For analysis of Foxp3-containing cells, a commercially available staining buffer set from eBioscience was used. Flow cytometric analysis was conducted on a BD LSR II flow cytometer, and data were analyzed using FlowJo 9.5.2 software (Tree Star, Ashland, OR).

ELISA

IFN-γ, IL-10, and IL-2 OptEIA ELISA kits were purchased from BD Biosciences. All ELISAs were performed by adhering to the manufacturer's instructions. Briefly, capture Ab (1:250 dilution) was coated overnight on MaxiSorp 96-well plates (Thermo Scientific, Rochester, NY) in coating buffer (0.1 M sodium carbonate or 0.1 M sodium phosphate). The following day, the plates were washed with PBS plus 0.05% Tween 20 and blocked with PBS plus 10% FBS for 1 h at room temperature. Next, the plates were washed again and incubated with supernatants from overnight cultures or from mouse sera for 2 h at room temperature. Following, the plates were washed again and incubated with a 1:250 dilution of capture Ab plus streptavidin-HRP conjugate solution for 1 h at room temperature. Finally, the plates were washed and incubated with substrate solution (tetramethylbenzidine; BD Biosciences) for 30 min in the dark. The reaction was stopped at this time with 1 M phosphoric acid. Absorbance was read on a Bio-Rad iMark microplate reader (Bio-Rad, Hercules, CA), and concentrations were calculated using a standard curve line of best fit on Microplate Manager Software (Bio-Rad). Mouse SAA ELISA kit was purchased from Immunology Consultants Laboratory (Portland, OR) and used as directed by the manufacturer's protocol. All reagents, including an Ab-precoated plate, were contained within the kit. For assessment of serum lactate dehydrogenase (LDH) activity, sera were diluted 1:10 in a 96-well microtiter plate (100 μl) in tandem with a 1:2 serial dilution (11,600–1.33 U/ml) of native bovine LDH standard (Cell Sciences, Canton, MA). Next, 100 μl 1:50 mix of two colorimetric reagents from a commercially available LDH assay kit (Clontech, Mountain View, CA) was added to the sera samples. Absorbance was measured every 10 min for 30 min at 490 nm (600-nm reference) and converted into activity units (U/ml) based on the prepared standard curve. Because LDH can be released from disrupted RBCs, any serum samples that showed visible signs of hemolysis were excluded from study.

Statistical analysis

Unpaired, two-tailed Student *t* tests were performed with $p < 0.05$ representing statistical significance in all figures except Fig. 1B (paired, two-tailed Student *t* test). Homogeneity of variance was determined by *F* test, with $F > 0.05$ as a threshold for equal variance. *F* tests were performed using Microsoft Excel 2010 software (Microsoft, Redmond, WA), and *p* values were determined using GraphPad Prism 6.01 (GraphPad Software, LaJolla, CA).

Results

RKIP drives IFN-γ production in mice undergoing SIRS

Systemic inflammatory response syndrome is associated with significant morbidity and mortality in patients, and results from the exorbitant release of inflammatory factors from immune cells (2). To study and model human SIRS in mice, we used a regimen involving multiple administrations of staphylococcal enterotoxin A (SEA), which induces potent T cell expansion by 48 h (35), and systemic proinflammatory cytokine production (Fig. 1A). To verify that we had indeed prompted systemic inflammation, serum from mice 90 min after the secondary administration of SEA was used to measure IFN-γ, IL-6, SAA, and LDH. These cytokines were chosen because they are considered prognostic markers for SIRS in human patients (36–39). As expected, significantly increased levels of IFN-γ, IL-6, SAA, and LDH were detected in sera after SEA (Fig. 1B).

To determine whether RKIP played a role in a SIRS response, we compared the cytokine output of SIRS and naive splenocytes restimulated in vitro with SEA from RKIP$^{-/-}$, wild-type littermates, and C57BL/6J mice at the height of the SIRS response. Splenocytes from all mice stimulated with SEA in vivo produced decreased levels of IL-2 when compared with naive controls, signifying that the T cells in these cultures were anergic or immunosuppressed similar to CARS T cells. However, the production of the effector cytokine IFN-γ was increased dramatically in wild-type mice relative to naive, but maintained or reduced in mice lacking RKIP, suggesting that RKIP may play a role in the optimal production of IFN-γ during a SIRS response (Fig. 1C). Fig. 1D shows that in each individual experiment the C57BL/6 splenocytes, from now on referred to as wild-type unless otherwise specified, produced substantially more IFN-γ in SIRS mice versus naive.

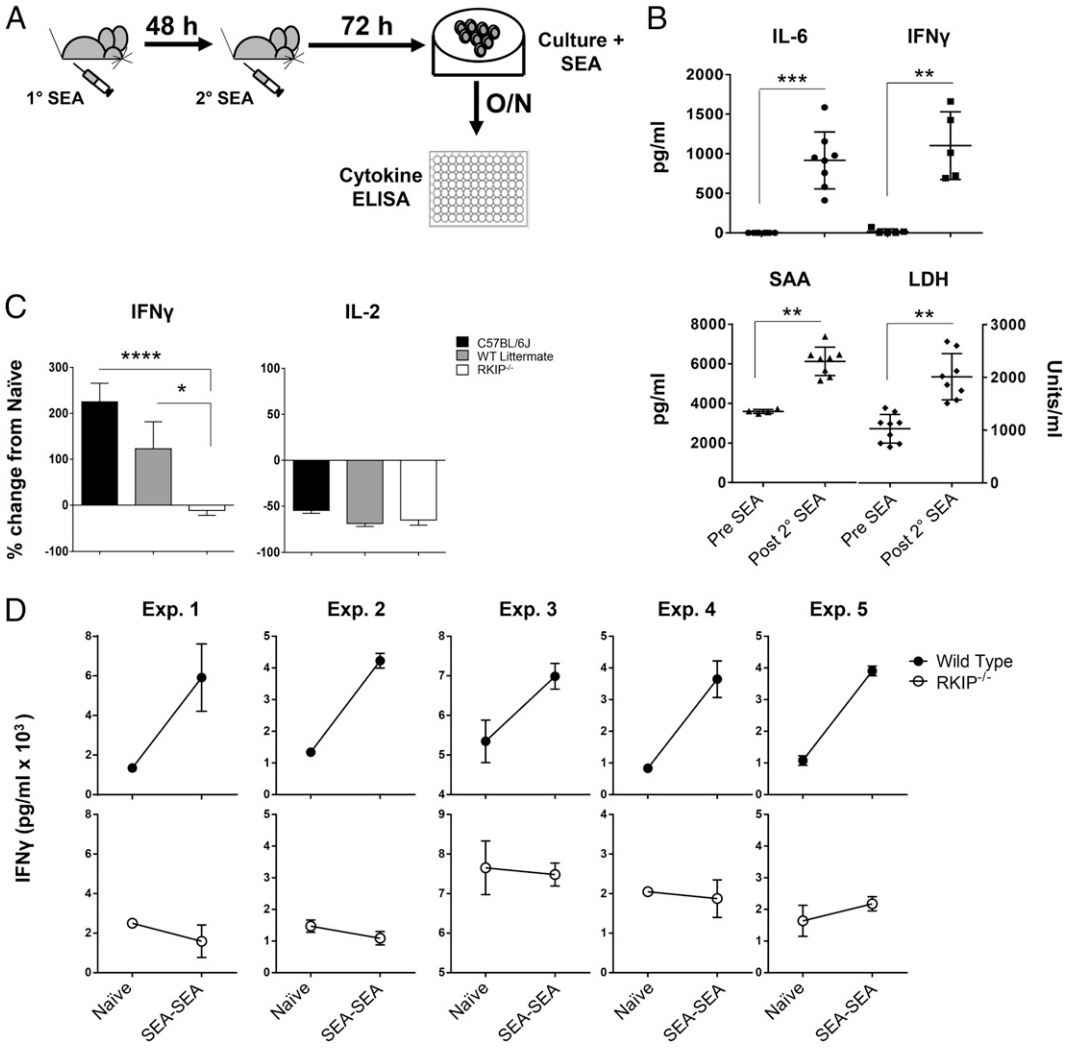

FIGURE 1. RKIP is necessary for optimal production of IFN-γ by splenocytes during SIRS. (**A**) Schematic of SIRS induction protocol. (**B**) Serum was isolated from C57BL/6 mice before 1° SEA and 90 min after 2° SEA treatment, and then quantified for IL-6, IFN-γ, SAA, and LDH by ELISA. Data are from three independent experiments, $n = 5–8$/group. (**C**) Splenocytes from C57BL/6J, wild-type littermate, and RKIP$^{-/-}$ mice were harvested 72 h after 2° SEA, as depicted in (A). A total of 5×10^5 cells/well was stimulated overnight with 0.1 μg SEA, and supernatants were collected and analyzed for IFN-γ and IL-2 by ELISA. Data are from five independent experiments, $n = 10–13$/group, and are expressed as a percent change from naive splenocytes cultured and stimulated similarly. (**D**) Absolute values of IFN-γ for C57BL/6J and RKIP$^{-/-}$ from each independent experiment in (C) with SEM. The *p* values were determined by unpaired *t* test between groups in (C) and (D) and by paired *t* test in (B), as follows: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

The RKIP$^{-/-}$ SIRS splenocytes did not make increased IFN-γ levels over their naive counterparts in this overnight culture.

To investigate the reason for reduced IFN-γ production seen in Fig. 1C and 1D, we phenotyped primary and secondary lymphoid organs from naive RKIP$^{-/-}$ mice and determined that there were no underlying deficits in T cell, B cell, NK cell, or CD11b^{+} APC populations (Supplemental Fig. 1A), as well as important thymic subsets (Supplemental Fig. 2A). Also, we found that there were no deficiencies in baseline cytokine production potential of IFN-γ in a multitude of lymphoid organs (Supplemental Fig. 1B). Next, we hypothesized that the reduced IFN-γ production could be due to a reduction in the expansion of SEA-specific (Vβ3^{+}) T cells. No difference in either SEA-specific CD8^{+} or CD4^{+} splenic T cells by percentage or total number after SIRS induction was detected (Fig. 2). Based on these results, we hypothesized that reduction in IFN-γ production might be T cell intrinsic.

SEA-specific CD8^{+} T cells are responsible for suboptimal IFN-γ production in RKIP$^{-/-}$ mice

To test our hypothesis suggesting that if the lower levels of IFN-γ were due to a defect in a specific cell type or if it was generalized among all splenocytes, we harvested splenocytes from naive and SIRS-induced RKIP$^{-/-}$ and wild-type mice and examined IFN-γ production in specific cell populations by flow cytometry after a short-term (4 h) in vitro restimulation with SEA (Fig. 3A). After SIRS induction, there was a marked decrease in the percentage of IFN-γ–producing CD8^{+} Vβ3^{+} T cells from RKIP$^{-/-}$ mice compared with wild type (Fig. 3B). Furthermore, of the CD8^{+} Vβ3^{+} T cells producing IFN-γ, RKIP$^{-/-}$ cells produced lower amounts compared with wild-type cells when using mean fluorescence intensity as a measurement (Fig. 3C). This defect in IFN-γ production was not observed in SEA-specific CD4^{+} T cells (Fig. 3B, 3C), bystander T cells, or splenic non-T cell populations (data not shown). Importantly, naive T cells did not produce IFN-γ in these short-term cultures, as opposed to the overnight stimulation in Fig. 1. Perhaps this is due to the IFN-γ gene locus not having sufficient time to open and translate IFN-γ mRNA into protein in 4 h. These data suggest that the suboptimal IFN-γ production seen in RKIP$^{-/-}$ SIRS splenocytes is due to a cell-intrinsic defect specifically in CD8^{+} Vβ3^{+} T cells.

Because of the reduced IFN-γ production by CD8^{+} Vβ3^{+} T cells, we tested whether higher levels of the anti-inflammatory cytokine IL-10 could explain this result, because there is a known reciprocity between these cytokines (40). IL-10 can be induced by *S. aureus* enterotoxins (41), and T regulatory cells (Tregs) proficiently synthesize this suppressive cytokine (42); thus, they were examined during SIRS in our model. Tregs from SIRS-induced RKIP$^{-/-}$ mice had a decreased propensity to synthesize IL-10

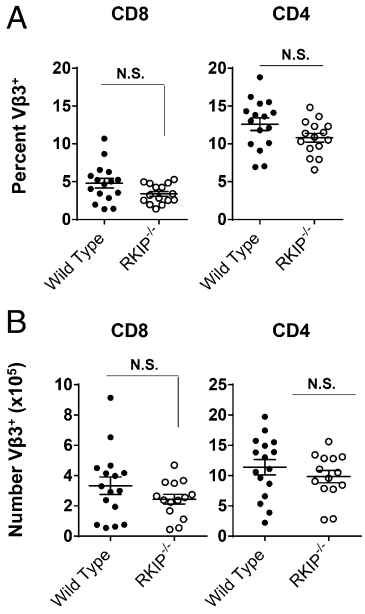

FIGURE 2. Reduced IFN-γ production in RKIP$^{-/-}$ mice during SIRS is not due to reduced expansion of SEA-specific Vβ3^{+} T cells. (**A**) Splenocytes from C57BL/6J and RKIP$^{-/-}$ mice were harvested 72 h after 2° SEA, as depicted in Fig. 1A, and stained directly ex vivo with Abs against CD3, CD4, CD8, and Vβ3; and CD3^{+}CD8^{+} or CD3^{+}CD4^{+} T cells were gated to determine the percentage of Vβ3^{+} T cells in spleen. (**B**) Total cell numbers of Vβ3^{+} T cells from spleen. Data in (A) and (B) are from five independent experiments, n = 15–16/group. Error bars represent SEM, and p values were determined by unpaired t test between groups.

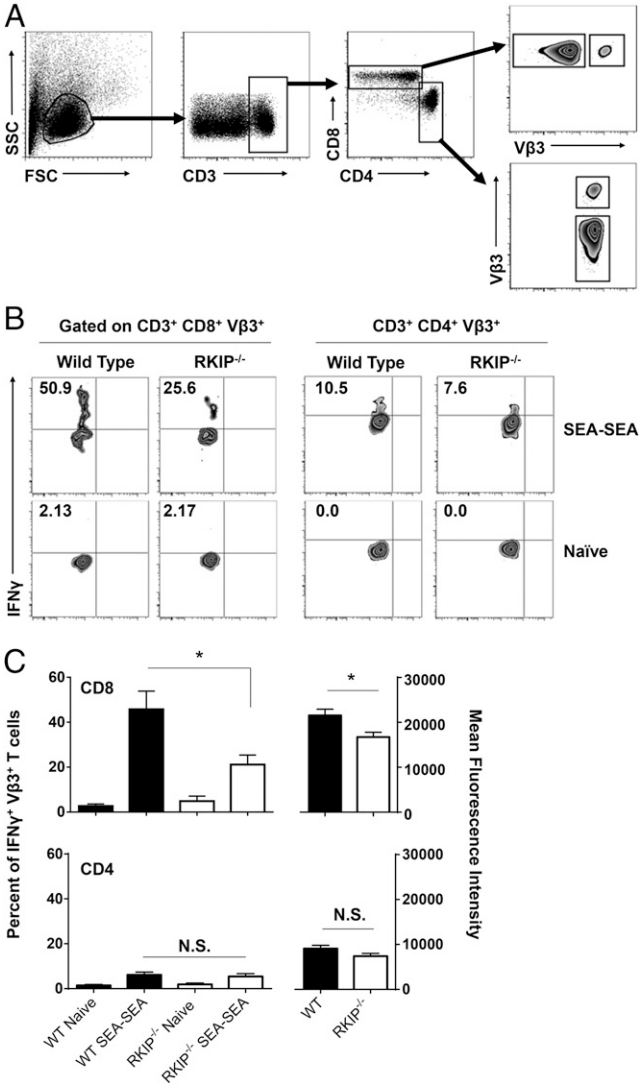

FIGURE 3. SEA-specific CD8^{+} effector T cells are responsible for suboptimal IFN-γ production in RKIP$^{-/-}$ mice. (**A**) Gating strategy for flow cytometric analysis used in (**B**) and (**C**). (B) Splenocytes were harvested, as described in Fig. 1A, and restimulated in vitro with 0.1 μg SEA/well plus brefeldin A for 4 h. After restimulation, cells were analyzed by intracellular cytokine staining for IFN-γ production. Data are plots from a representative experiment displaying median values. (C) The *left panels* show the mean ± SEM of IFN-γ–producing T cells, and *right panels* display mean fluorescence intensity of IFN-γ^{+} T cells. Data are from three independent experiments, n = 8 mice/group. Error bars represent SEM, and p values were determined by unpaired t test between groups, $^{*}p < 0.05$.

during stimulation with PMA plus ionomycin when compared with wild-type controls (Fig. 4A, *left panel*). This was not due to a difference in the percentage of Tregs between groups (Fig. 4A, *right panel*) or a baseline alteration in IL-10 production potential from naive Tregs (Fig. 4B). Interestingly, when whole splenocytes from RKIP$^{-/-}$ and wild-type mice were restimulated with SEA and compared for IL-10 induction, a slight, but not statistically significant, reduction was seen in the RKIP$^{-/-}$ group (Fig. 4C). This implies that other cell types within the spleen have the ability to compensate, to a degree, for the loss of IL-10 production in Tregs during SIRS-associated inflammation.

SIRS CD8$^+$ RKIP$^{-/-}$ T cells have an intrinsic signaling defect that lies downstream of the TCR

To better localize the lesion that was responsible for suboptimal IFN-γ production from RKIP$^{-/-}$ CD8$^+$ Vβ3$^+$ T cells after TCR triggering, we wanted to determine whether these cells ever had the potential to make IFN-γ or not. Therefore, we tested RKIP$^{-/-}$ and wild-type splenocytes after initiation of SIRS with PMA plus ionomycin restimulation as opposed to SEA. The defect in IFN-γ production by RKIP$^{-/-}$ SIRS CD8$^+$ Vβ3$^+$ T cells was no longer apparent (Fig. 5A, *upper panel*). Furthermore, there was also no longer a difference in the release or secretion of IFN-γ between RKIP$^{-/-}$ and wild-type splenocytes after PMA plus ionomycin stimulation (Fig. 5B). Hence, the signaling defect seen in RKIP$^{-/-}$ SIRS CD8$^+$, but not SIRS CD4$^+$, T cells most likely lies downstream of TCR engagement, but upstream of the factors induced by Ca^{2+} influx and phorbol esters.

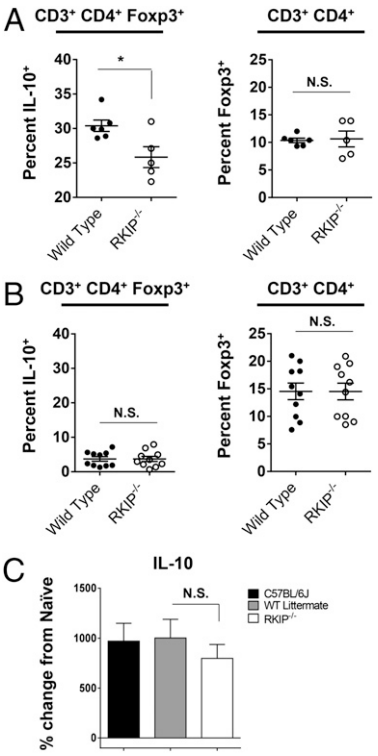

FIGURE 4. Tregs from RKIP$^{-/-}$ mice during SIRS show reduced IL-10 production. (**A**) Splenocytes were harvested, as outlined in Fig. 1A, and restimulated in vitro with PMA plus ionomycin, including brefeldin A, for 4 h. After restimulation, cells were surfaced phenotyped with Abs against CD3, CD4, and Foxp3, and stained intracellularly for IL-10 production; cells were then analyzed by flow cytometry. Data are from two independent experiments, n = 5–6/group. (**B**) Splenocytes were harvested from 4- and 11-wk-old naive RKIP$^{-/-}$ mice or wild-type littermates (see Supplemental Fig. 1). A total of 5×10^5 cells/well was stimulated in vitro with PMA plus ionomycin, including brefeldin A, for 4 h; then surface phenotyped with Abs specific to CD3, CD4, and Foxp3; and stained intracellularly for IL-10 production. Data are from three independent experiments, n = 10/group. (**C**) Supernatants from Fig. 1 were analyzed for IL-10 production by ELISA. Data are expressed as a percentage change from naive splenocytes cultured and stimulated similarly. As in Fig. 1, data are from five independent experiments, n = 10–13/group. Error bars represent SEM, and p values determined by unpaired t test between groups, *$p < 0.05$.

Blockade of RKIP using the small molecule inhibitor locostatin greatly diminishes IFN-γ production from wild-type CD8$^+$ T cells in SIRS

To test the therapeutic potential of RKIP blockade in SIRS, we restimulated 48 h post-SIRS induction splenocytes from wild-type mice overnight with SEA or PMA plus ionomycin in the presence of locostatin or vehicle control. The optimal effective dose of locostatin was chosen based on titration studies that elicited a dose that showed a biological effect while leaving the vast majority of T cells viable, even after overnight stimulation (Supplemental Fig. 3A, 3B). As in RKIP$^{-/-}$ SIRS splenocytes, inhibition of RKIP function with locostatin significantly decreased IFN-γ production after TCR retriggering with SEA, but not after PMA plus ionomycin restimulation, suggesting once again that RKIP is playing a role downstream of the TCR (Fig. 5C).

Because SEA cross-links T cells with APCs, we investigated whether the loss of IFN-γ production was due to poor APC presentation. First, we assessed expression of MHC II and the costimulatory molecules CD80/CD86 on splenic APC populations and found no difference between RKIP$^{-/-}$ and wild-type littermate controls (Supplemental Fig. 4A, 4B). Additionally, we isolated APCs and CD8$^+$ T cells from the spleens of RKIP$^{-/-}$ and wild-type littermate mice 48 h after SIRS induction. We then restimulated cultures of RKIP$^{-/-}$ APCs and wild-type (or RKIP$^{-/-}$) CD8$^+$ T cells overnight with SEA, and, unlike stimulation of the intact splenocyte population (Fig. 1), we observed no difference in IFN-γ production (data not shown). Thus, to rule out any unappreciated developmental defect that may have confounded these results, we isolated APCs and CD8$^+$ T cells from spleens of C57BL/6 mice 48 h after SIRS induction and treated them separately with either locostatin or vehicle in vitro for 1 h. Cells were washed twice to remove residual locostatin or vehicle, and untreated APCs or CD8$^+$ T cells were added and restimulated overnight with SEA. We observed that by pretreating wild-type CD8$^+$ SIRS T cells with locostatin, IFN-γ production was diminished to 30% of untreated or vehicle-treated controls (Fig. 5D). However, blocking RKIP in APCs before restimulation had little effect of IFN-γ output, once again suggesting that RKIP is playing a role at the level of the T cell and can be therapeutically targeted to diminish IFN-γ responses from CD8$^+$ T cells during SIRS.

RKIP$^{-/-}$ T cells retain the ability to mount an effective response to PAMP-TLR–mediated stimuli

Because RKIP$^{-/-}$ SIRS CD8$^+$ T cells have an intrinsic defect downstream of TCR triggering, we hypothesized that RKIP$^{-/-}$ cells may also have difficulty with proliferation, survival, and cytokine production during a vaccination response. To address this question, we replaced the second in vivo administration of SEA in our SIRS induction model with the vaccine adjuvant LPS (Fig. 6A). This model mimics a standard vaccination protocol that results in the expansion and survival of SEA-specific T cells, which become Th1-like cells (33). We found no difference in the survival of CD4$^+$ Vβ3$^+$ or CD8$^+$ Vβ3$^+$ T cells between RKIP$^{-/-}$ and wild type in terms of percentage or total number at day 12 after SEA-LPS immunization. This was seen in both spleen (Fig. 6B, Table I) and liver (Fig. 6D, Table I), as well as in pooled

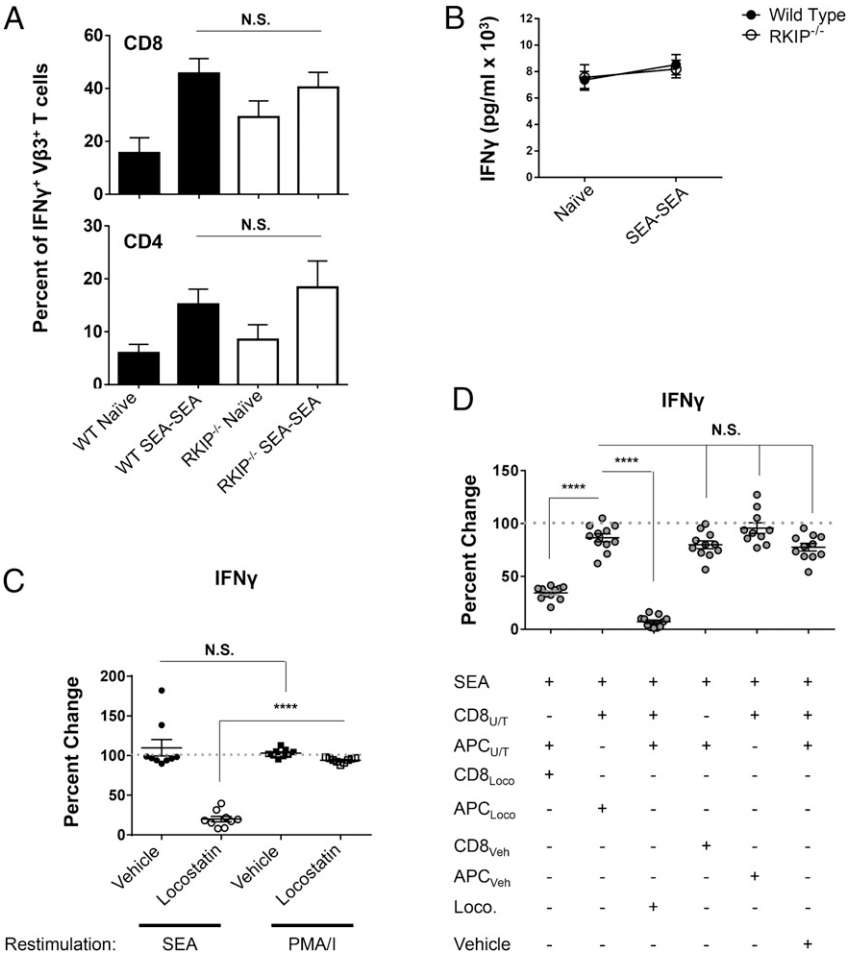

FIGURE 5. SEA-specific $CD8^+$ effector T cells lacking RKIP have an intrinsic signaling defect that lies downstream of the TCR. (**A**) Splenocytes from Fig. 3 were restimulated in vitro for 4 h with PMA plus ionomycin, including brefeldin A, and then stained with Abs against CD3, CD4, CD8, Vβ3, and IFN-γ, followed by flow cytometric analysis. Data are from three independent experiments, n = 8/group. (**B**) Splenocytes were harvested as in (A) and cultured overnight (5×10^5/well) with PMA + ionomycin. Subsequently, supernatants were collected and analyzed for IFN-γ production by ELISA. Data are from two independent experiments, n = 6/group. (**C**) Forty-eight hours post-SIRS induction, splenocytes from C57BL/6J mice were isolated and restimulated overnight with SEA or PMA plus ionomycin in the presence of media alone, vehicle alone (DMSO), or 5 μM locostatin. The supernatants from these cultures were then analyzed for IFN-γ production by ELISA. The gray dotted line represents SEA (or PMA + ionomycin) restimulation without locostatin or vehicle. Data are from three independent experiments, n = 9/group. (**D**) Isolated and magnetic bead–purified APC and $CD8^+$ T cell populations from C57BL/6J mice 48 h after SIRS induction were treated separately with either 5 μM locostatin or vehicle for 1 h. Subsequently, cells were washed twice with media to remove any residual locostatin or vehicle present, and then untreated APCs or $CD8^+$ T cells (in a 1:1 ratio) were added to these cultures and restimulated overnight with 0.1 μg SEA. As controls, cultures of untreated APCs and $CD8^+$ T cells containing locostatin or vehicle for the entire duration were stimulated in parallel. The supernatants of these overnight cultures were then assessed for IFN-γ production by ELISA. The gray dotted line represents untreated APCs and $CD8^+$ T cells stimulated with SEA alone. Data are from three independent experiments, n = 10–11/group. Error bars represent SEM, and p values determined by unpaired t test between groups.

peripheral lymph nodes (Table I). Also, there were no observable differences in contraction of SEA-specific cells after SEA immunization alone (Table I). These data are consistent with the lack of changes in clonal expansion during the SIRS model seen in Fig. 2. Unexpectedly, when cells were harvested from the spleen and liver at day 12 postimmunization and restimulated in vitro with SEA overnight, $RKIP^{-/-}$ cells now made equal or greater amounts of IFN-γ (Fig. 6C, 6E). These results were also recapitulated when cells were restimulated with plate-bound anti-CD3 acting as the TCR trigger instead of SEA, although a slight reduction in IL-2 production was seen in this instance (Supplemental Fig. 2B). Importantly, these data suggest that, although $RKIP^{-/-}$ $CD8^+$ T cells have a defect in effector cytokine production during SIRS-mediated inflammation, they can still expand, survive, and contribute to effector cytokine production in response to PAMP-TLR–mediated reactions.

Discussion

SIRS is an extensive and profound burden on the United States healthcare system (4). One well-known cause of SIRS induction is exposure to superantigens, most famously TSST-1 enterotoxin from *S. aureus*, which was directly responsible for many cases of toxic shock syndrome in the 1980s. TSST-1 and SEA bypass canonical Ag processing and activate a large percentage of the T cell repertoire by cross-linking β-chain V regions, specifically Vβ3, of the TCR to constant domains of the MHC on APCs (43). T cells are critically important for mediating SIRS because they can directly release proinflammatory factors themselves and potentiate the inflammatory and destructive effects of innate immune cells (13). In fact, IFN-γ, an important T cell effector cytokine, has been shown to be necessary for certain clinical sequelae of SIRS, including acute lung injury (16). By utilizing a T cell–dependent model of SIRS, we sought to identify proteins that could alter

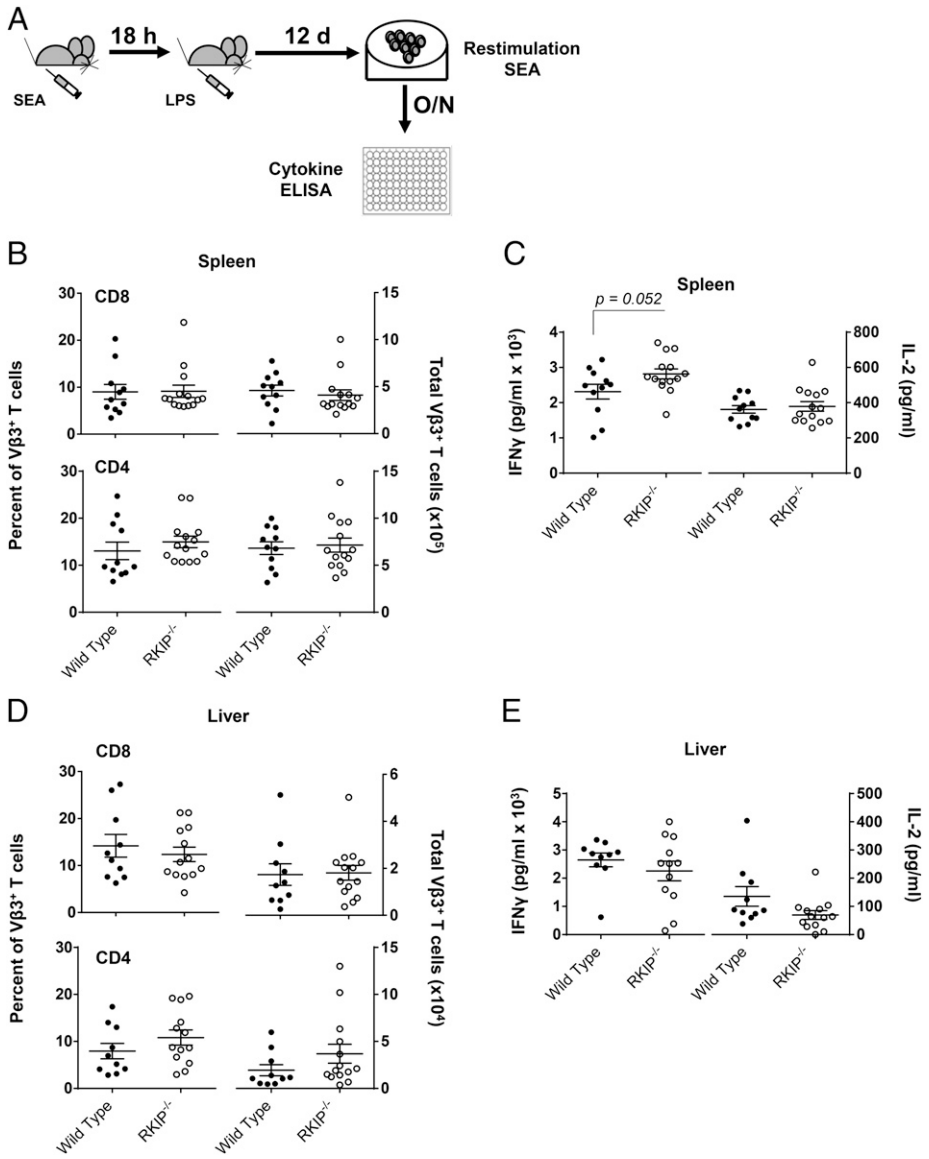

FIGURE 6. RKIP$^{-/-}$ T cells can expand, survive, and contribute to cytokine production in response to PAMP-TLR–mediated stimuli. (**A**) Schematic representation of immunization protocol. (**B**) Splenocytes were harvested at 12 d post-immunization with LPS, as outlined in (A), and stained directly ex vivo for SEA-specific (Vβ3$^+$) T cells, as outlined in Fig. 3A. Data are from three independent experiments, n = 11–14/group. (**C**) Splenocytes were harvested as in (A) and cultured (5×10^5/well) overnight with 0.1 μg SEA. The next day, IFN-γ and IL-2 levels in supernatants were measured by ELISA. Data are from three independent experiments, n = 11–14/group. (**D**) Liver leukocytes were harvested, treated, and analyzed as in (B). (**E**) Liver leukocytes were harvested, treated, and analyzed as in (C). Error bars represent SEM, and p values determined by unpaired t test between groups.

cytokine production in T cells, thus lessening the inflammatory response at several different levels. We show that RKIP represents a new, and potentially valuable, therapeutic target because its inhibition curbs IFN-γ synthesis without shutting down responses to PAMPs. From a mechanistic perspective, we demonstrate that CD8$^+$ T cells may be a spring for cytokine production during a SIRS response, and have demonstrated that RKIP is coupled to continued IFN-γ potential in anergic or immunosuppressed CD8$^+$ T cells.

However, like all animal models, SEA exposure does not recapitulate all aspects of human SIRS perfectly. As with many inflammatory mouse models, especially ones on the C57BL/6 genetic background, most are resistant to the typical symptomology seen in human SIRS patients (i.e., fever, lethargy, malaise, hypovolemia, organ dysfunction, death). This may be due to inflammatory reactions in humans and mice eliciting different genetic responses to burns, trauma, or endotoxemia, all of which trigger

Table I. Normal clonal expansion, contraction, and survival in RKIP$^{-/-}$ mice after SEA-LPS immunization

		pLN		Spleen		Liver	
		CD8[a]	CD4[a]	CD8	CD4	CD8	CD4
Naive	Wild type	2.61 ± 0.20	4.30 ± 0.31	2.99 ± 0.46	4.07 ± 0.18	2.44 ± 0.44	1.76 ± 0.57
	RKIP$^{-/-}$	2.32 ± 0.13	4.26 ± 0.12	2.80 ± 0.27	5.69 ± 0.93	2.38 ± 1.19	1.69 ± 0.46
LPS	Wild type	2.70 ± 0.20	4.25 ± 0.13	3.20 ± 0.82	4.98 ± 0.72	2.17 ± 0.28	1.90 ± 0.88
	RKIP$^{-/-}$	2.29 ± 0.09	3.98 ± 0.60	3.27 ± 0.61	5.48 ± 0.52	2.25 ± 0.94	1.53 ± 0.13
SEA	Wild type	1.14 ± 0.19	2.03 ± 0.13	2.78 ± 0.92	3.43 ± 0.25	1.59 ± 0.27	0.96 ± 0.15
	RKIP$^{-/-}$	1.44 ± 0.19	2.20 ± 0.17	2.03 ± 0.15	3.61 ± 0.31	1.75 ± 0.26	1.67 ± 0.29
SEA + LPS	Wild type	5.36 ± 1.02	7.57 ± 1.04	9.00 ± 1.57	13.04 ± 1.86	14.18 ± 2.31	7.95 ± 1.55
	RKIP$^{-/-}$	5.87 ± 0.68	8.73 ± 0.78	9.13 ± 1.31	14.96 ± 1.23	12.37 ± 1.49	10.82 ± 1.55

Data are a pooled analysis of all experiments in Fig. 6. Three independent experiments, n = 11–14/group.
[a]Percentage of Vβ3$^+$ T cells in various tissues on day 12 after no treatment, LPS, SEA, or both.
pLN, Peripheral lymph node.

SIRS (44). Nevertheless, we feel that this model provides at least a reasonable starting point to analyze potential molecular targets that can modify systemic inflammatory responses.

The NF-κB and MAPK pathways are both critical for the production of proinflammatory cytokines (21, 22), and RKIP has been previously shown to be a negative regulator of these pathways. RKIP imparts control by interfering with the kinase activities of several signaling factors in these cascades. Little is known regarding the in vivo effects of RKIP, especially within the immune system. Based on the findings of these previous studies, we anticipated that mice lacking RKIP would be more prone to exaggerated cytokine production during T cell activation, but, contrary to our hypothesis, we found that RKIP was actually important for enhancing IFN-γ production in $CD8^+$ SIRS T cells after serial triggering of the TCR with SEA (Figs. 1, 3). This is critical because SEA induces a SIRS response in mice that results in T cells that are anergic [characterized by their failure to produce IL-2 (45)], but can continue to perpetuate inflammation due to their ability to make IFN-γ in large quantities. On a molecular level, we showed that RKIP is playing a role in the signaling machinery downstream of the TCR, as evidenced by the fact that the diminished IFN-γ production from $CD8^+$ T cells lacking RKIP was rescued if the TCR is bypassed using PMA and ionomycin (Fig. 5). Interestingly, expansion of these T cells, which is another critical process mediated by MAPK signaling, was unaltered in our model of SIRS (Fig. 2). This implies that RKIP's role within effector T cells may be more critical for cytokine output rather than proliferation.

Currently, no choice drug exists for the treatment of SIRS. Therapy focuses on treating specific infections, if one is present, in combination with therapies that stabilize the respiratory and cardiovascular systems if a patient has signs of shock (10). Clinical trials centered on antagonizing the function of TNF-α and IL-1β failed to show efficacy (46); however, our data suggest that perhaps inhibiting the effects of IFN-γ may be a better therapeutic strategy. The only treatment to target the robust inflammation of SIRS that has shown marginally better outcomes is the usage of low-dose steroids like hydrocortisone (47). This therapeutic approach is broadly immunosuppressive, thereby minimizing inflammatory reactions that are advantageous to the host, such as productive responses to vaccines or pathogens. This is critical for a patient pool that might be afflicted with established bacterial, viral, or fungal infections. Nevertheless, this is in contrast to patients suffering from autoimmunity in which steroid-mediated suppression of the immune system would provide beneficial effects with a diminished risk of infection compared with SIRS. However, an ideal therapeutic for either scenario would be one that dampens the inflammatory effects of T cells that are being chronically stimulated through the TCR (e.g., enterotoxins in SIRS or self-Ag in autoimmunity), while leaving advantageous immune responses, such as to a vaccine or new infection, largely intact.

RKIP may be one potential molecule that, if targeted, could possibly achieve these optimal therapeutic goals. For example, when RKIP is absent from the immune system, or when therapeutically targeted, effector T cells, serially triggered through their TCR, produce significantly less IFN-γ than wild type, while impinging only marginally on overall IL-10 production (Fig. 4). In addition, when the TCR is engaged only once in vivo and adjuvanted with TLR stimulation from LPS, splenocytes synthesize normal levels of IFN-γ (Fig. 6). Thus, it is possible that blockade of RKIP could diminish IFN-γ production from effector T cells during SIRS, while permitting a relatively unabated response to PAMPs. However, it remains to be determined what would occur in a complex response in which both serial TCR triggering and PAMP-TLR–mediated stimuli both exist simultaneously or what the exact role, if any, that RKIP plays in these responses. Finally, therapeutically targeting RKIP may provide a substantial benefit over direct inhibition of IFN-γ because it allows for the alleviation of IFN-γ effects at the level of synthesis rather than receptor binding. This may be especially important in acute SIRS in which it could be too late to impact disease outcomes once IFN-γ has been produced.

Although our data suggest that a loss of RKIP leads to a T cell–intrinsic defect in optimal IFN-γ production, it still does not exclude the possibility of potential T cell–extrinsic effects as well. In fact, in 2006, Schuierer et al. (48) showed that RKIP expression may play a role in appropriate macrophage and dendritic cell differentiation. Because APCs are critical for the activation of T cells in response to classically presented Ags, as well as superantigens, any deficit in APC function could also impart effects onto cytokine production from T cells. We show that RKIP is playing a role at the level of the $CD8^+$ T cell in responses to superantigens because inhibition of RKIP in APCs specifically had no effect on IFN-γ production, but inhibition in $CD8^+$ T cells did (Fig. 5D). Also, if T cell–extrinsic effects such as this were playing a large role in our model systems, we would have expected to see little or no response in our SEA-LPS studies, which was not the case. However, this does not explicitly rule out a potential extrinsic defect in response to MHC-restricted peptide Ags that must undergo canonical processing and presentation within APCs.

Another facet that makes RKIP a unique therapeutic target is that it is a druggable protein. A small molecule inhibitor of RKIP, locostatin, is available (49). Locostatin exerts its inhibitory effects on RKIP by alkylating a conserved histidine residue (His86) within its ligand-binding pocket (29). Modification of this residue prevents RKIP from binding to its aforementioned ligands, thus preventing their inhibition. In addition to abating IFN-γ production from wild-type cells during SIRS (Fig. 5C), locostatin also potently blocks IFN-γ and TNF-α production upon triggering mouse OT-I or human Ag-specific T cells against influenza with cognate peptide and human PBMCs treated with LPS (50). However, the specificity of this inhibitor is still being investigated as it has several potential off-target effects (49). Furthermore, the target analyses for locostatin have largely been conducted in immortalized cell lines (51), and thus, the exact mechanism of how it mediates its inhibitory effects within an in vivo immunological system remains unclear. This allows significant room for improvement in developing better target-specific inhibitors of RKIP before use in a clinical setting. Nevertheless, our new data pinpoint a locostatin effect on $CD8^+$ T cells, but not APCs, in response to the pathogenic enterotoxin SEA (Fig. 5D). In sum, our data suggest that RKIP represents a potentially new therapeutic target for reducing the effects of IFN-γ from $CD8^+$ effector T cells during the serial TCR-triggering events in SIRS.

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Disclosures

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