JLB

Brief Conclusive Report

Fas regulates neutrophil lifespan during viral and bacterial infection

Joanne A. O'Donnell,[*,†] Catherine L. Kennedy,[‡] Marc Pellegrini,*[,†] Cameron J. Nowell,* Jian-Guo Zhang,*[,†] Lorraine A. O'Reilly,*[,†] Louise Cengia,* Stuart Dias,[§] Seth L. Masters,*[,†] Elizabeth L. Hartland,[‡] Andrew W. Roberts,*[,†,¶] Motti Gerlic,*[,†,‖] and Ben A. Croker*[,†,§,1]

*Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia; [†]Department of Medical Biology and [¶]Faculty of Medicine, University of Melbourne, Parkville, Victoria, Australia; [‡]Department of Microbiology and Immunology, University of Melbourne at Peter Doherty Institute for Infection and Immunity, Melbourne, Victoria, Australia; [§]Division of Hematology/Oncology, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA; and [‖]Department of Clinical Microbiology and Immunology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

RECEIVED NOVEMBER 12, 2013; REVISED SEPTEMBER 11, 2014; ACCEPTED SEPTEMBER 29, 2014. DOI: 10.1189/jlb.3AB1113-594RR

ABSTRACT

The regulation of neutrophil lifespan is critical for a circumscribed immune response. Neutrophils are sensitive to Fas/CD95 death receptor signaling in vitro, but it is unknown if Fas regulates neutrophil lifespan in vivo. We hypothesized that FasL-expressing CD8$^+$ T cells, which kill antigen-stimulated T cells during chronic viral infection, can also induce neutrophil death in tissues during infection. With the use of *LysM-Cre Fas$^{fl/fl}$* mice, which lack *Fas* expression in macrophages and neutrophils, we show that Fas regulates neutrophil lifespan during lymphocytic choriomeningitis virus (LCMV) infection in the lung, peripheral blood, and spleen. Fas also contributed to the regulation of neutrophil numbers in the colon of *Citrobacter rodentium*-infected mice. To examine the effects of infection on Fas activation in neutrophils, we primed neutrophils with TLR ligands or IL-18, resulting in ablation of Fas death receptor signaling. These data provide the first in vivo genetic evidence that neutrophil lifespan is controlled by death receptor signaling and provide a mechanism to account for neutrophil resistance to Fas stimulation during infection.
***J. Leukoc. Biol.* 97: 321–326; 2015.**

Introduction

Neutropenia is an important predictor of poor outcome in patients with disseminated infection [1], yet our understanding of the factors that control neutrophil lifespan during infection and inflammation in vivo remains limited. Approximately 100 billion neutrophils are produced daily at the steady state [2], and this increases during emergency granulopoiesis. Elevated neutrophil production must be matched by alterations in neutrophil lifespan and clearance of neutrophils by phagocytes to prevent the pathologic accumulation of neutrophils in tissues. Genetic studies demonstrate that neutrophil viability is controlled by Bim but not Bax, Bcl-2, or Bcl-w [3]. The prosurvival protein Mcl-1 is a key regulator of neutrophil survival at steady state and during inflammation [4]. Neutrophil lifespan can be increased by cytokines, such as G-CSF and GM-CSF, as well as TLR ligands [5–7], and changes in median lifespan correlate with expression of antiapoptotic regulators, including Mcl-1, Bcl-2, A1, and the proapoptotic regulator Bad [3, 8]. Key roles for neutrophils have been proposed for systemic inflammatory response syndrome, acute respiratory distress syndrome, inflammatory bowel disease, cystic fibrosis, and viral infections, including CMV, influenza, and respiratory syncytial virus (RSV), suggesting that aberrant regulation of neutrophil viability may contribute to pathology [9–13]. Despite these observations, a lack of neutrophil-specific and/or inducible conditional gene-targeting approaches has precluded genetic analysis of the regulators of neutrophil viability in vivo during infection.

Fas (CD95), a cell-surface death receptor that is activated by membrane-bound or soluble FasL, is proposed to mediate neutrophil survival in vivo [14–16]; however, the physiologic relevance of these studies has not been formally tested. Fas can induce the release of IL-1β from macrophages and neutrophils in vivo, independently of caspase-1 [17, 18]. Activation of Fas leads to the formation of a DISC with FADD and procaspase-8, resulting in caspase-8 activation [19]. Caspase-8 can directly cleave and activate the effector caspase-3 and -7 or cleave proapoptotic Bid to activate Bak and Bax to induce mitochondrial outer-membrane permeabilization, leading to activation of effector caspases and cell death [19]. A number of negative

Abbreviations: $^{-/-}$ = deficient, c-FLIP = cellular Fas-associated protein with death domain-like IL-1β-converting enzyme-inhibitor protein, DISC = death-inducing signaling complex, FADD = Fas-associated protein with death domain, FasL = Fas ligand, LCMV = lymphocytic choriomeningitis virus, LTA = lipoteichoic acid, *Ly5.1* = *Ptprc^a Pep3^b*, *Ly5.2* = *Ptprc^b Pep3^a*, $Pam_{3/2}CSK_4$ = palmitoyl-3/2-cysteine-serine-lysine-4, PI = propidium iodide, WT = wild-type

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. Correspondence: Boston Children's Hospital, 300 Longwood Ave., CLS3003, Boston, MA 02115, USA. E-mail: ben.croker@childrens.harvard.edu

regulators of Fas-induced death have been proposed, including c-FLIP, which inhibits caspase-8 activation [20].

We have previously reported that Bcl-2 and Mcl-1 inhibit Fas-induced apoptosis in neutrophils ex vivo [21]. Here, we continue our studies on the negative regulators of Fas signaling in neutrophils and the physiologic role of Fas in regulating median neutrophil lifespan in vivo. Key roles in host defense and inflammation have been ascribed to neutrophils following bacterial and viral infection [11–13], and in this study, we use two models of infection known to be regulated by Fas: LCMV and *C. rodentium* infection. We demonstrate, for the first time in vivo, that neutrophil lifespan is controlled by Fas during viral and bacterial infection and that TLR ligands and IL-18 protect neutrophils from Fas-induced death in vitro in a dose-dependent manner. The protection of neutrophils occurs independently of Bcl-2 prosurvival family members.

Many viral infections are complicated by secondary bacterial infection. This study demonstrates that a viral infection can induce neutrophilic inflammation in the lung, liver, spleen, and blood. This study also provides ex vivo data showing how neutrophils can "ignore" Fas death signals to deal with a subsequent secondary bacterial infection. Taken together, these data suggest a mechanism for neutrophils to survive and respond to primary viral infection and likely to secondary bacterial infection in tissues with an abundance of proapoptotic, FasL-expressing $CD8^+$ T cells.

MATERIALS AND METHODS

Mice

C57BL/6, ubiquitin-GFP [22], $FasL^{lpr}$ [23], *LysM-Cre* [24], and $Fas^{fl/fl}$ [25] mice were generated on a C57BL/6J background or backcrossed to C57BL/6J mice for >10 generations. All experiments were carried out in accordance with institute animal ethics guidelines and approval.

Bone marrow chimeras

For hematopoietic reconstitution experiments, congenic C57BL/6.SJL *Ly5.1* mice were reconstituted with 10^6 C57BL/6J *Ly5.2* fetal liver cells from $Bak^{-/-}$ $Bax^{-/-}$ E13.5 embryos after 2, 5.5-Gy doses of irradiation, given 3 h apart. For mixed bone marrow chimeras, irradiated Ly5.1 mice were reconstituted with 2.5×10^6 bone marrow cells from ubiquitin-GFP or Ly5.1/5.2 mice, combined with *LysM-Cre* $Fas^{fl/fl}$ at a 1:1 ratio.

Neutrophil purification

Neutrophils were prepared as described previously [26]. The purity of neutrophil preparations was routinely >98%, as assessed by cytology following May-Grünwald Giemsa staining.

Apoptosis assays

Cells were stimulated with recombinant FcFasL (a trimeric form of FasL) protein purified from FcFasL-transfected HEK-293 cell culture supernatant by affinity chromatography using the MabSelect resin (GE Healthcare, Waukesha, WI), followed by size-exclusion chromatography on a Superdex 200 column (GE Healthcare). The cell line was provided by Pascal Schneider (University of Lausanne, Switzerland). Neutrophils were primed with GM-CSF (1 h) before treatment with LPS (10 ng/ml) or IL-18 (10 ng/ml). Neutrophil viability was measured by live cell imaging by use of PI as described [21].

LCMV infection

Mice were infected by i.v. injection of 2×10^6 PFU LCMV docile.

C. rodentium infection

Mice were inoculated with 5×10^8 CFU of *C. rodentium* strain ICC169 from an overnight culture in 200 μl PBS via oral gavage [27]. Colonization was monitored by serial dilution of freshly collected feces plated onto Luria agar, supplemented with 50 μg/ml nalidixic acid.

Hematology and flow cytometry

Automated cell counts were performed on blood collected from the retro-orbital plexus into Microtainer tubes containing EDTA (Sarstedt, Nümbrecht, Germany) by use of an Advia 2120 hematologic analyzer (Siemens, Munich, Germany). Flow cytometric analysis of hematopoietic cells was performed on a LSRFortessa cell analyzer (BD Biosciences, San Jose, CA, USA).

Genotyping

Sorted cells were genotyped for the deleted *Fas* allele by use of the following primers: 5′-CCTTCCATTGATGGACAGTTC-3′ and 5′-TTTCCACACTT-TGGTCTTCG-3′.

Statistics

Unless otherwise specified, data are presented as mean ± 1 SD or 1 SEM. Comparisons were performed by use of Student's *t*-test or 2-way ANOVA with Bonferroni's post hoc test.

RESULTS AND DISCUSSION

Fas does not regulate neutrophil viability at steady state

Previous studies of $Fas^{lpr/lpr}$ and $FasL^{gld/gld}$ mice reported that Fas does not regulate neutrophil lifespan at the steady state or during acute inflammation induced by thioglycolate [28, 29]. More recently, *LysM-Cre* was used to delete a conditional allele of *Fas*, resulting in altered monocytopoiesis, increased dendritic cell activation, and an accumulation of autoreactive B cells, suggesting that expression of Fas in myeloid cells was essential to prevent systemic lupus erythematosus-like disease [30]. Abnormalities in neutrophil development and lifespan were not reported in this study. To investigate a role for Fas in the regulation of neutrophil lifespan in vivo, we characterized *LysM-Cre* $Fas^{fl/fl}$ mice, where *LysM-Cre* is widely used to target gene ablation, specifically to myeloid cells [24]. *LysM-Cre* $Fas^{fl/fl}$ neutrophils are resistant to FasL-induced death (**Fig. 1A**). However, routine analysis of the specificity of *LysM-Cre* expression in these mice revealed Fas deletion in purified B lymphocytes but not T lymphocytes (Fig. 1B). This suggests that the autoimmune phenotype, developing in 6- to 8-month-old *LysM-Cre* $Fas^{fl/fl}$ mice [30], may also be a consequence of a small population of B cells that expresses *LysM-Cre* and expands with age. Moreover, previous studies have demonstrated that FasL-treated neutrophils and macrophages can process IL-1β and IL-18 in vitro [17, 18, 31], precluding the use of *LysM-Cre* $Fas^{fl/fl}$ mice as a model for assessing the role of Fas in the neutrophil lifespan during infection, as any changes in IL-1β and IL-18 would confound the interpretation of data from *LysM-Cre* $Fas^{fl/fl}$ mice. Although caspase-1 is likely the dominant player in IL-1β production in *C. rodentium*-infected mice [32, 33], a role for Fas and caspase-8 cannot be excluded [34, 35].

To circumvent these issues, we created mixed bone marrow chimeras containing equal numbers of WT and *LysM-Cre* $Fas^{fl/fl}$ hematopoietic cells in lethally irradiated hosts. These chimeric mice enabled us to examine the cell-intrinsic defects in

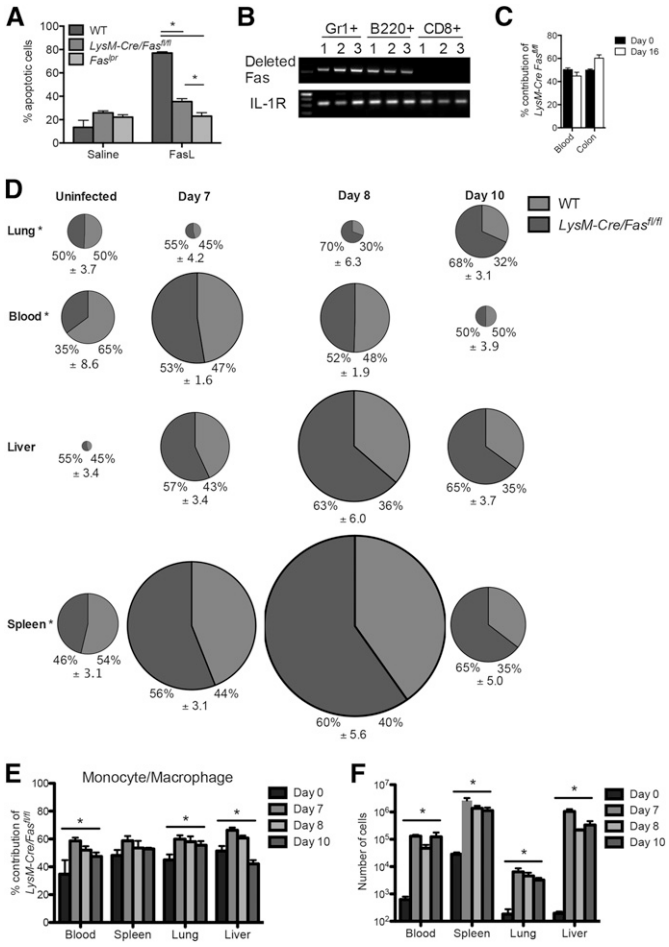

Figure 1. Fas contributes to neutrophil and macrophage lifespan during *Citrobacter* and LCMV infection. (A) Viability of *LysM-Cre Fas*$^{fl/fl}$, *Fas*$^{lpr/lpr}$, and WT neutrophils, 3 h after FasL stimulation. *$P < 0.05$, mean ± SD from 2–3 independent samples. (B) Fas is deleted in B lymphocytes. PCR genotyping for the deleted *Fas* allele indicates *LysM-Cre*-mediated excision in Gr1$^+$ and B220$^+$ but not CD8$^+$ cells from *LysM-Cre Fas*$^{fl/fl}$ mice. Purified cells from 3 independent mice are shown. *Il1r*-specific primers were used as a control for DNA integrity. (C) Contribution of *LysM-Cre Fas*$^{fl/fl}$ neutrophils in the peripheral blood and colon during *C. rodentium* infection ($n = 3$–12 mice). *$P < 0.05$ by Student's *t*-test. (D) Contribution of *LysM-Cre Fas*$^{fl/fl}$ neutrophils during acute LCMV infection. Pie charts represent relative neutrophil numbers in the lung, blood, liver, and spleen in uninfected mice or on d 7, 8, and 10 postinfection in lethally irradiated mice reconstituted with *LysM-Cre Fas*$^{fl/fl}$ and WT bone marrow at a 1:1 ratio. Each pie chart shows percent contribution ± 1 SEM of *LysM-Cre Fas*$^{fl/fl}$ and WT neutrophils; $n = 4$ mice/time-point. *$P < 0.05$ by 2-way ANOVA. Data shown are representative of duplicate experiments on independent groups of mice. (E) Contribution of *LysM-Cre Fas*$^{fl/fl}$ blood monocytes and lung, liver, and spleen macrophages in lethally irradiated mice reconstituted with *LysM-Cre Fas*$^{fl/fl}$ and WT bone marrow at a 1:1 ratio on d 0, 7, 8, and 10 of LCMV infection. Mean ± 1 SEM; $n = 4$ mice/time-point. *$P < 0.05$ by 2-way ANOVA. (F) CD8$^+$ T lymphocytes are recruited to the blood, spleen, lung, and liver during LCMV infection. Mean ± 1 SEM; $n = 4$ mice/time-point. *$P < 0.05$ by 2-way ANOVA.

neutrophil and macrophage lifespan mediated by Fas without the confounding effects of autoimmune disease and would expose WT and Fas-deficient neutrophils equally to any IL-1β and IL-18 present in the extracellular environment. In chimeric mice, WT and *LysM-Cre Fas*$^{fl/fl}$ neutrophils and macrophages were present at a 1:1 ratio in the lung, peripheral blood, spleen, and liver, 8 weeks after reconstitution (Fig. 1D and E), indicating that Fas does not regulate neutrophil or macrophage lifespan at steady state.

Neutrophil accumulation in the colon during *Citrobacter* infection is regulated by Fas

Severe neutrophil infiltration penetrating across the muscularis mucosa and epithelial erosion associated with increased colon weight, crypt height, and severe diarrhea have been reported in *Fas*$^{lpr/lpr}$, *Fas*$^{gld/gld}$, and *Bid*$^{-/-}$ mice infected with the enteropathogenic-like mouse pathogen *C. rodentium* [34]. We hypothesized that a Fas-deficiency in neutrophils prolonged neutrophil lifespan and contributed to the accumulation of neutrophils in the colon. To test this, we infected WT:*LysM-Cre Fas*$^{fl/fl}$ chimeric mice with *C. rodentium* and examined the proportion of neutrophils in the colon and peripheral blood at d 0 and 16 postinfection. We observed a significant increase in the proportion of *LysM-Cre Fas*$^{fl/fl}$ neutrophils in the colon but not peripheral blood at d 16 (Fig. 1C), indicating that Fas regulates neutrophil lifespan during bacterial infection. However, it was clear that other cell death pathways must also contribute to the regulation of neutrophil lifespan in the colon during *C. rodentium* infection. Pearson et al. [34] reported that the type III secretion system effector protein NleB from *C. rodentium* actively inhibits Fas signaling via an interaction with FADD, TNFR type 1-associated death domain protein and receptor-interacting serine/threonine-protein kinase 1 and inhibition of caspase-8 signaling. Hence, it is possible that the lifespan of WT neutrophils is also actively prolonged by *C. rodentium* via the inhibition of death-receptor signaling.

Fas regulates the lifespan of macrophages and neutrophils during LCMV infection

We reasoned that Fas regulates neutrophil survival, where FasL-expressing lymphocytes and NK cells are present but not at steady state when these cells are absent. LCMV infection stimulates the expansion of FasL-expressing cytotoxic CD8$^+$ T lymphocytes in the blood, lung, liver, and spleen (Fig. 1F), as well as neutrophil recruitment in these tissues (Fig. 1D). LCMV-infected WT:*LysM-Cre Fas*$^{fl/fl}$ chimeric mice had a significant increase in the contribution of *LysM-Cre Fas*$^{fl/fl}$ monocytes in the blood and macrophages in the liver and lungs at d 7, 8, and 10 (Fig. 1E), indicating that Fas mediates monocyte and macrophage lifespan during LCMV infection. *LysM-Cre Fas*$^{fl/fl}$ neutrophils preferentially accumulated in the blood, liver, and spleen at d 7, 8, and 10 post-LCMV infection, indicating that Fas also regulates the lifespan of neutrophils during LCMV infection (Fig. 1D). Furthermore, an additional increase in the contribution of *LysM-Cre Fas*$^{fl/fl}$ neutrophils was observed during the clearance phase of infection between d 8 and 10, suggesting that FasL-induced cell death plays a key role in regulating neutrophil clearance in the tissues. It is possible that *LysM-Cre Fas*$^{fl/fl}$ neutrophils that escape *LysM-Cre*-mediated deletion (thereby retaining *Fas* expression) are killed more efficiently than Fas-deficient neutrophils, so the actual contribution of Fas

to neutrophil clearance may be even greater than what we have reported.

It is possible that Fas contributes to neutrophil migration; however, previous investigators have discounted a role for Fas in neutrophil extravasation following thioglycolate administration [28]. The normal recruitment of Fas-deficient neutrophils to the liver and spleen during LCMV infection (Fig. 1D) and the rapid induction of apoptosis by FasL [21] make it unlikely that Fas controls neutrophil migration during *C. rodentium* or LCMV infection.

IL-18 and TLR ligands inhibit Fas-induced neutrophil apoptosis

During infection, neutrophils encounter numerous host- and pathogen-derived factors that influence their lifespan, including death-inducing FasL-expressing T cells. Thus, we reasoned that neutrophils must be capable of avoiding FasL-induced death in settings where neutrophil function is required to contain infection. We used a previously described live cell imaging approach [21] to monitor neutrophil viability over time and obtain kinetic data to determine whether Fas-induced apoptosis of neutrophils is influenced by IL-18 or TLR ligands, which delay spontaneous apoptosis in neutrophils [5–7]. PI was used to monitor neutrophil viability, which has previously been shown to be sufficient to monitor the rapid induction of neutrophil apoptosis in response to FasL [21]. Treatment of neutrophils with the MyD88-dependent cytokine IL-18 or the TLR ligands Pam_3CSK_4, Pam_2CSK_4, or LPS strongly protected neutrophils against Fas-induced death (**Fig. 2A** and **B** and Supplemental Fig. 1). High doses of FasL overcame this protective effect and induced neutrophil death (Fig. 2B). To define further the parameters of LPS- and IL-18-mediated inhibition of FasL-induced cell death, neutrophils were exposed to FasL before treatment with LPS or IL-18 (Fig. 2C). Both LPS and IL-18 protect neutrophils from FasL-induced cell death as late as 1 h after exposure to FasL, indicating that neutrophils encountering FasL are not destined to undergo cell death.

The intrinsic apoptosis pathway does not mediate the effects of LPS and IL-18

To investigate further the negative regulation of FasL-induced death of neutrophils, we pretreated neutrophils with an inhibitor of protein synthesis. The blocking of protein synthesis with cycloheximide partially inhibited the protective effect of LPS and IL-18 (Fig. 2D and Supplemental Fig. 1B), indicating that protein synthesis is required for protection from FasL. Prosurvival Bcl-2 family proteins, which act upstream of the mediators of apoptosis Bak and Bax, can delay Fas-induced apoptosis in neutrophils [21], and their expression can be regulated by TLR ligands. Therefore, we used $Bak^{-/-}Bax^{-/-}$ neutrophils, which are protected from death via the intrinsic apoptosis pathway, to determine if alterations in the activity of the Bcl-2-regulated intrinsic death pathway mediate the protective effects of IL-18 and LPS. $Bak^{-/-}Bax^{-/-}$ neutrophils undergo rapid cell death in response to Fas stimulation, and death is prevented upon IL-18 and LPS treatment (**Fig. 3A**). Specifically, complete protection of $Bak^{-/-}Bax^{-/-}$ neutrophils by LPS or IL-18 pretreatment compared with FasL treatment alone indicates that any increase in expression of regulators of Bak and Bax, such as Bcl-2 or Mcl-1, cannot be responsible for this inhibitory effect and is not a result of alterations in the intrinsic death pathway. LTA, a TLR2 ligand, was capable of inhibiting caspase-8 processing in response to Fas activation, demonstrating that the inhibition of Fas death receptor signaling by MyD88-dependent ligands occurs at the level of the DISC (Fig. 3B).

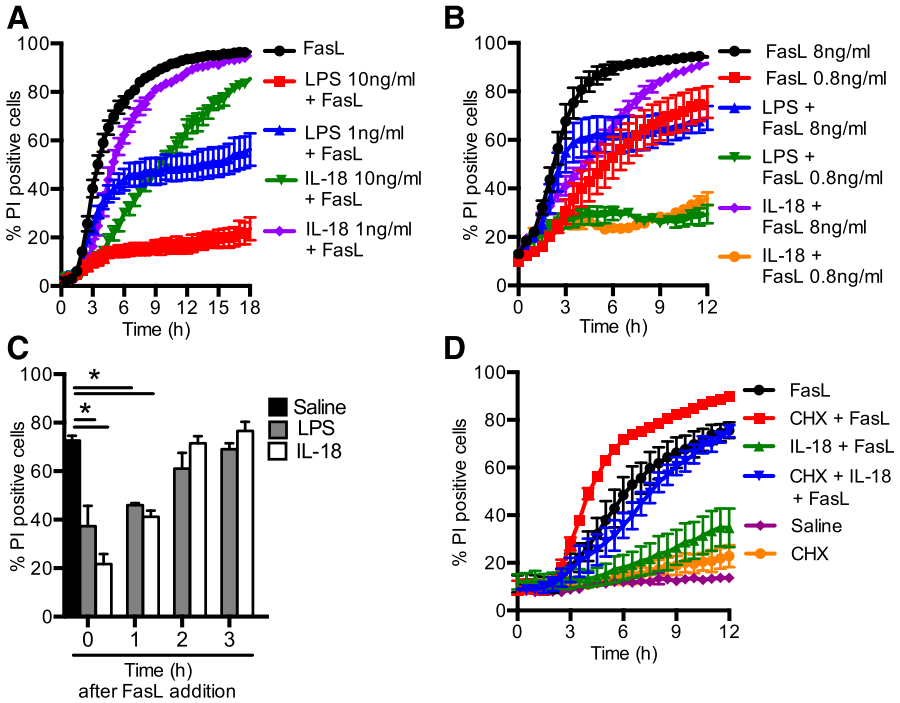

Figure 2. LPS and IL-18 inhibit Fas-induced neutrophil death. (A and B) MyD88-dependent stimuli inhibit FasL-induced apoptosis in a dose-dependent manner. Neutrophils were primed for 1 h with LPS or IL-18 and then stimulated with FcFasL. Cell viability was monitored by use of PI. Mean ± 1 SEM from 3 independent samples, performed in 1 experiment. Data shown are representative of 2 experiments. (C) LPS and IL-18 inhibit Fas-induced death after neutrophils encounter FasL. Neutrophils were treated with FcFasL at the same time as LPS or IL-18 (10 ng/ml) or 1, 2, or 3 h after FasL. Cell viability was determined at 5 h by use of PI. $*P < 0.05$; $n = 3$, mean ± 1 SEM from 3 independent samples, tested in 1 experiment. Data shown are representative of 2 experiments. (D) The protective effects of IL-18 are partially dependent on protein synthesis. Cycloheximide (CHX; 0.1 ng/ml) was added 15 min before LPS or IL-18 and FcFasL.

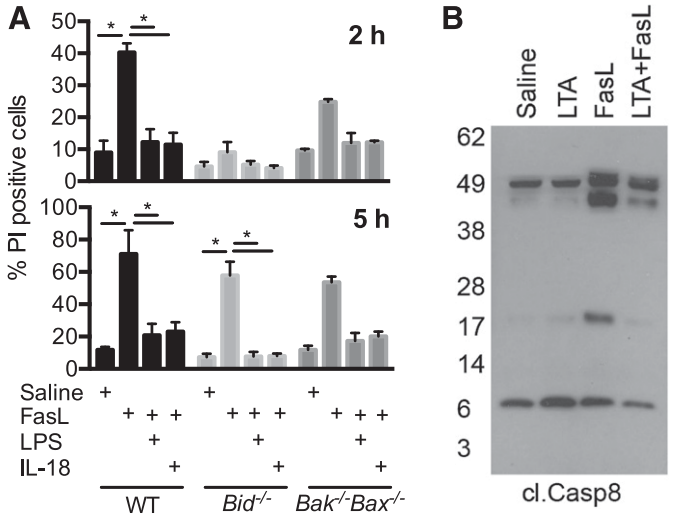

Figure 3. IL-18 and LPS regulate FasL-induced cell death upstream of the Bcl-2-regulated intrinsic apoptosis pathway. *Bid*$^{-/-}$ or *Bak*$^{-/-}$ and *Bax*$^{-/-}$ do not alter the protective effects of IL-18 or LPS. Neutrophils were treated with IL-18 or LPS (10 ng/ml) before FcFasL (1/250). Cell viability was determined by use of PI. Mean ± 1 SEM from 3 WT, 3 *Bid*$^{-/-}$, or 2 *Bak*$^{-/-}$*Bax*$^{-/-}$-independent samples, analyzed in 1 experiment. *$P < 0.05$. (B) FasL-induced caspase-8 cleavage is (cl.Casp8) inhibited by MyD88-dependent stimuli. Bone marrow neutrophils were primed with LTA for 1 h before activation with cross-linked His-tagged FasL.

These genetic data show that Fas contributes to the lifespan of neutrophils in vivo during infection. These data also demonstrate that in addition to Fas, other currently undefined proteins must play critical roles in controlling neutrophil lifespan. Perforin maintains dendritic cell homeostasis in synergy with Fas, suggesting that a similar mechanism may control the lifespan of neutrophils [36, 37]. Alternatively, the intrinsic apoptosis pathway may control neutrophil survival during LCMV infection. For example, deficiency of the proapoptotic protein Bim leads to increased neutrophil numbers in vivo [38], and both Bim and Fas contribute to T cell clearance during chronic murine gamma herpesvirus infection [39]. The intrinsic apoptosis pathway may integrate with the extrinsic apoptosis pathway by negatively regulating Fas-induced cell death via transcriptional or post-translational regulation of the prosurvival proteins Bcl-2 and Mcl-1, as we have demonstrated previously [21].

MyD88-dependent signals emanating from TLR1/2, TLR2/6, TLR4, and the IL-18R are potent negative regulators of Fas signaling in neutrophils. c-FLIP is induced by MyD88- and NF-κB-dependent signaling [40], and we hypothesize that c-FLIP is an important negative regulator of Fas signaling in neutrophils. The failure of Fas- [41], MyD88-, IL-18-, and TLR2-deficient mice to clear LCMV infection [42, 43] suggests that MyD88-dependent induction of c-FLIP and negative regulation of Fas signaling and caspase-8 activation in myeloid and lymphoid cells [40] may be required to modulate innate and adaptive immune responses.

Concluding remarks

These data demonstrate that the median lifespan of neutrophils is sensitive to apoptosis induced by Fas. However, Fas is not the only regulator of neutrophil survival during LCMV infection. Neutrophils receive multiple inputs from pathways, such as the intrinsic apoptosis pathway and perhaps nonapoptotic necroptotic and pyroptotic death pathways, to control the lifespan of neutrophil subsets. In addition to previous reports of cross-talk between intrinsic and extrinsic cell death pathways in neutrophils [21], these data demonstrate that IL-18 and the detection of pathogens by neutrophils can cross-talk with the extrinsic death pathway and reprogram neutrophils otherwise destined to die by Fas-mediated apoptosis during viral infection and secondary bacterial infection.

AUTHORSHIP

J.A.O. and C.L.K. designed and performed the experiments, analyzed the data, and wrote the manuscript. M.G., M.P., C.J.N., S.L.M., E.L.H., and A.W.R. provided intellectual input and reviewed the manuscript. L.C. conducted the research. B.A.C. and M.G. designed the experiments, analyzed the data, and wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by project grants (637367, 1045532, and 1009145), program grants (461219, 1016647, 461221, 1016701, and 606788), a Australian National Health and Medical Research Council (NHMRC) Practitioner Fellowship to A.W.R. (637309), a Dora Lush Scholarship (to J.A.O.), a Discovery Early Career Researcher Award (to C.L.K), and an Independent Research Institutes Infrastructure Support Scheme Grant (361646) from the NHMRC; fellowships are from the Australian Research Council (to B.A.C.); the Leukemia and Lymphoma Society (SCOR Grants 7413 and 7001-13); and a Victorian State Government Operational Infrastructure Support Grant. The authors thank A. Strasser, B.T. Kile, and T. Mak for mice, J. Corbin for excellent technical assistance and E. Lanera and K. Scicluna for outstanding animal husbandry.

DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:
apoptosis · TLR · IL-18 · LCMV · *Citrobacter rodentium*