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Cutting Edge: Adaptive Versus Innate Receptor Signals Selectively Control the Pool Sizes of Murine IFN-γ– or IL-17–Producing γδ T Cells upon Infection

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Introduction

Immunosurveillance in the lymphoid system and in peripheral tissues involves key nonredundant contributions by γδ T cells (1, 2). The widespread implication of γδ T cells is often attributed to the cells' rapid and abundant production of IFN-γ in mice and in humans (3–5). However, γδ T cells are also a critical source of IL-17A (referred to hereafter as IL-17) in animal models of infection (6–8) or autoimmune disorders (9–12). There is therefore a pressing need to understand what signals regulate the activation and functional response of γδ T cells.

Although our ignorance of most TCRγδ ligands confounds the full dissection of peripheral γδ T cell activation, some γδ T cells have been shown to respond in vivo to innate receptor ligands, notably those for the activating NKG2D receptor (13). This concept was recently extended by reports of TCR-independent stimulation of IL-17 production by IL-1β plus IL-23 (12) and TLR2 or Dectin-1 ligands (14). Collectively, these data have raised the question of the relative importance of innate immunity-associated receptors versus the somatically recombined TCR and its accessory receptors for the activation of γδ T cells in vivo.

We have investigated this key question by employing mouse infection models, coupled with our recently described classification of thymic and peripheral γδ T cell populations (15). We show that for the IFN-γ–producing, $CD27^+$ γδ ($γδ^{27+}$) subset, CD27 costimulation synergizes with the TCR to provide survival and proliferative signals that determine their expansion upon viral or parasitic infection in vivo. By contrast, the peripheral pool size of IL-17–producing γδ T cells, which lack CD27 expression ($γδ^{27-}$), depends on TLR/MyD88-

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dependent innate immune signals during malaria infection. Thus, discrete functional subsets of γδ T cells can be differentially controlled by adaptive and innate immune receptors.

Materials and Methods

Mice

All mice were adults 4–10 wk of age. C57BL/6 (B6), B6.Thy1.1, B6.TCRα-deficient, B6.TCRδ-deficient mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6.CD27-deficient mice (16) were a kind gift from Dr. Jannie Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Mice were bred and maintained in the specific pathogen-free animal facilities of Instituto de Medicina Molecular (Lisbon, Portugal), Instituto Gulbenkian de Ciência (Oeiras, Portugal), and Cancer Research UK (London, U.K.). All experiments involving animals were performed in compliance with the relevant laws and institutional guidelines and have been approved by the local ethics committees.

mAbs

Anti-mouse mAbs specific for TCRδ (GL3), IFN-γ (XMG1.2), CD3ε (145.2C11), and Vγ4 (UC3-10A6) were purchased from BD Pharmingen (San Diego, CA). Anti-CD27 (LG.7F9), CD19 (eBio1D3), IL-17A (eBio17B7), CD11b (M1/70), and CD11c (N418) mAbs were purchased from eBioscience (San Diego, CA). Anti-Vγ1 (2.11) mAb was a kind gift from Pablo Pereira (Institut Pasteur, Paris, France).

Flow cytometry

All FACS-based assays were performed as previously described (15). Cells were sorted on an FACSAria (BD Biosciences, San Jose, CA) and analyzed on FACSCanto or FACSCalibur (BD Biosciences).

Cell culture and in vitro assays

Cells were cultured as described (15) and stimulated with anti-CD3ε mAb, either plate-bound (1 or 10 μg/ml) or soluble (0.5 μg/ml), in the presence of 10^5 mitomycin C-treated (Sigma-Aldrich, St. Louis, MO) splenocytes. Where indicated, the following reagents were added to the medium: soluble rCD70 (kindly provided by Dr. Jannie Borst) or LPS from *Salmonella* Minnesota R595 (10 μg/ml; Alexis Biochemicals, Plymouth Meeting, PA).

Immunoblotting

Cells were lysed in RIPA buffer complemented with a complete Protease Inhibitor Cocktail (Roche Mini Tablet, Roche Applied Science, Burgess Hill, U.K.). Total protein lysates were boiled in sample loading buffer (LDS, Invitrogen, Carlsbad, CA) containing 100 mM DTT. Proteins were subjected to electrophoresis using NuPage 4–12% Bis-Tris precasted gels (Invitrogen) and transferred onto a polyvinylidene fluoride membrane (GE Healthcare, Piscataway, NJ). Membranes were stained with rabbit anti-p100/p52 (4882, Cell Signaling Technology, Beverly, MA), mouse anti-GAPDH (MAB374, Millipore, Bedford, MA), donkey anti-rabbit IgG (31458, Thermo Scientific, Waltham, MA), or goat anti-mouse IgG + IgM (31446, Thermo Scientific). Proteins were detected using Super Signal West Femto substrate (Thermo Scientific).

Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described (15) using the following primers: *Bcl2a1*-Fwd, 5′-AATAACACAGGAGAATGGATACGG-3′ and *Bcl2a1*-Rev, 5′-TCTCTGGTCCGTAGTGTTACTTGA-3′; *Efa1*-Fwd, 5′-

ACACGTAGATTCCGGCAAGT-3′ and *Efa1*-Rev, 5′-AGGAGCCCTTTCCCATCTC-3′; *Ccnd2*-Fwd, 5′-CACCGACAACTCTGTGAAGC-3′ and *Ccnd2*-Rev, 5′-TCCACTTCAGCTTACCCAACA-3′; *Cdk4*-Fwd, 5′-GCCAGAGATGGAGGAGTCTG-3′ and *Cdk4*-Rev, 5′-CCTTGTGCAGGTAGGAGTGC-3′; *Cdk6*-Fwd, 5′-CGAGTGCAGACCAGTGAGG-3′ and *Cdk6*-Rev, 5′-TGTGCACACATCAAACAACCT-3′; *Il1b*-Fwd, 5′-GGACAGAATATCAACCAACAAGTGATA-3′ and *Il1b*-Rev, 5′-GTGTGCCGTCTTTCATTACACAG-3′; and *Il23*-Fwd, 5′-TCCCTACTAGGACTCAGCCAAC-3′ and *Il23*-Rev, 5′-GCTGCCACTGCTGACTAGAA-3′.

Adoptive cell transfers

FACS-sorted cells were injected i.v. into B6.TCRδ-deficient mice (3×10^5 cells/mouse). Mice were sacrificed, and splenocytes were collected after 5 d.

Injection of TCR or TLR agonists

Mice were injected i.p. with 50–100 μg anti-CD3 mAb (145.2C11) or 50 μg LPS from *Salmonella* Minnesota R595 (Alexis Biochemicals) or 50 μg $Pam_3CysSerLys_4$ (PAM; InvivoGen, San Diego, CA). Cells from the peritoneal cavity (PEC) were collected for analysis after 3 d.

Malaria infection

Mice were infected i.v. with 10^6 GFP-transgenic *Plasmodium berghei* ANKA-infected RBCs and monitored as previously described (15). Splenocytes were collected for analysis after 3 d.

Murid herpesvirus-4 infection

Mice were infected i.p. with 10^6 PFU murid herpesvirus-4 (MuHV-4), and cells from the PEC were collected after 8 d. Infection was confirmed by ex vivo reactivation assays, as previously described (17).

Statistical analysis

Statistical significance of differences between populations was assessed using Student *t* test and is indicated as NS ($p \geq 0.05$), *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Results and Discussion

CD27 costimulation provides survival and proliferative signals to γδ T cells

When purified TCRγδ$^+$ CD3$^+$ CD27$^+$ (γδ^{27+}) and TCRγδ$^+$ CD3$^+$ CD27$^-$ (γδ^{27-}) lymph node (LN) and splenic cells were activated in vitro with anti-CD3ε mAb, γδ^{27+} cells proliferated substantially, displaying a cycling profile and a blasting morphology, whereas γδ^{27-} cells remained quiescent and were highly prone to TCR/CD3-induced apoptosis in vitro and in vivo (Fig. 1*A*, 1*B*, Supplemental Fig. 1). Of note, TCR stimulation did not significantly modify the repertoire of either γδ T cell subset (Supplemental Fig. 2).

To directly test whether CD27 regulates the response to TCRγδ stimulation, we supplemented anti-CD3–treated cultures of total γδ cells with soluble rCD70 (sCD70), which acts as a CD27 agonist (18). There was a dose-dependent increase in the live/dead cell ratio among proliferating γδ cells (Fig. 1*C*), and this was associated with an accumulation of IFN-γ and TNF-α (Fig. 1*D*), signature cytokines of γδ^{27+} cells (15).

To gain mechanistic insight into this effect, biochemical and transcriptional events implicated in lymphocyte survival and proliferation were investigated. In B cells, the TNF ligand family member, BLyS (also known as BAFF), is known to decrease cell death by cleaving NF-κB p100, which is induced by Ag receptor signaling, to p52 (19, 20). Likewise, the high levels of p100 in TCR-stimulated $\gamma\delta^{27+}$ cells were reduced by sCD70 treatment, with concomitant accumulation of p52 (Fig. 1*E*). Furthermore, sCD70 potentiated TCR-mediated induction of antiapoptotic (*Bcl2a1*) and cell cycling (*Cyclin D2*, *CDK4*, and *CDK6*) genes in $Cd27^{+/+}$, but not in $Cd27^{-/-}$ γδ cells, whereas sCD70 treatment in the absence of TCR stimulation had no effect (Fig. 1*F* and data not shown). These results point to the TCR–CD27 axis as a fundamental regulator of the survival and proliferation of $\gamma\delta^{27+}$ cells and imply a striking parallel between CD27 costimulation of γδ T cells and BAFF-R costimulation of B cells.

CD27 signals are required for the expansion of IFN-γ–producing γδ $^{27+}$ cells

To assess if CD27 costimulation was necessary for optimal γδ T cell expansion in vitro and in vivo, competition assays were performed between CD27-deficient (Thy1.2) and wild-type (WT) Thy1.1 congenic γδ splenocytes. Cells were mixed at a 1:1 ratio and either cultured with anti-CD3 in the presence of APCs or injected into TCRδ-deficient hosts. There was a marked advantage of $Cd27^{+/+}$ γδ cells in expansion in both scenarios (Fig. 2*A*, 2*B*, Supplemental Fig. 3). Next, the impact of CD27 deficiency on the γδ T cell response to infection was examined. Given that $\gamma\delta^{27+}$ cells are functionally committed to IFN-γ production (15), immune responses to herpesviruses are critically dependent on IFN-γ (21), and γδ lymphocytes have been repeatedly implicated in controlling infections with human (22) and murine (23) herpesviruses, we infected mice with MuHV-4. MuHV-4 infection resulted in the accumulation of IFN-γ–producing, but not of IL-17–producing, γδ cells in the PEC of WT animals (Fig. 2*C*). Critically, this was dependent on CD27 (Fig. 2*C*), as was a similar expansion of IFN-γ–producing γδ splenocytes in a malaria infection model (Fig. 2*D*). Of note, CD27 expression was essentially stable postinfection in adoptively transferred γδ cells (Supplemental Fig. 4). Collectively, these data establish the importance of CD27 for determining the peripheral pool size of IFN-γ–producing γδ cells upon infection. The general significance of these findings is indicated by evidence that CD27 signaling likewise selectively promotes αβ Th1 cell differentiation (24).

Innate TLR/MyD88-dependent signals selectively expand IL-17–producing γδ $^{27-}$ cells in vivo

Because IL-17–producing γδ T cells do not express CD27 (15), other signals must account for their documented expansion in response to various infectious organisms (6–8, 15). In this regard, Mills and collaborators (12) recently showed that TLR agonists, in particular LPS, can stimulate dendritic cells to produce IL-1β and IL-23, which jointly promote IL-17 production by γδ cells in vitro, in the absence of TCR activation (12). To examine whether these results might apply in vivo, LPS was initially injected into the PEC of WT or TLR4-deficient mice, and γδ cells were retrieved and analyzed 72 h later. LPS treatment led to a marked and selective proliferation of $\gamma\delta^{27-}$ cells in WT but not TLR4-deficient mice (Fig. 3*A*, Supplemental Fig. 5). Similar data were obtained using the TLR2 agonist PAM, with a concomitant increase of γδ PEC cells producing IL-17, but not IFN-γ (Supplemental Fig. 6). Of note, we obtained no evidence for direct stimulation of $\gamma\delta^{27-}$ cells by LPS or PAM in vitro (Supplemental Fig. 7), whereas isolated IL-17–producing $\gamma\delta^{27-}$ cells responded strongly to IL-1β and IL-23, for which candidate sources in vivo (postinfection) were $CD11b^{hi}CD11c^{lo}$ myeloid cells (Supplemental Fig. 8).

Furthermore, $\gamma\delta^{27-}$ splenocyte expansion to malaria infection was significantly reduced in MyD88-deficient animals (Fig. 3*B*), in which both TLR2 and TLR4 pathways are blocked

(25). This resulted in a complete failure to expand IL-17–producing γδ cells (Fig. 3*C*), whereas the pool of IFN-γ–producing γδ cells was unaffected (not shown). Collectively, our results demonstrate that innate TLR/MyD88-dependent signals selectively control the peripheral pool size of murine IL-17–producing γδ cells. The general significance of these findings is implied by recent evidence for a role of TLRs in the differentiation of IL-17–producing $CD4^+$ (26) and $CD8^+$ (27) αβ T cells.

In sum, the γδ cell response to infection constitutes a primary example of how a T cell compartment can be composed of two arms, respectively regulated by innate versus adaptive immunity components. For murine lymphoid γδ T cells, the separation of these two arms begins in the thymus, where commitment to IFN-γ or IL-17 expression occurs (10, 15, 28). This developmental preprogramming of γδ cells may be crucial for their rapid responses upon activation in the periphery, as γδ T cell responses to infectious organisms can immediately deploy differentiated effector cells, which simply need to increase in number (2). This may also explain that CD27, a coreceptor typically associated with the clonal expansion of adaptive αβ T cells (16), can account for the rapid expansion of first line of defense IFN-γ–producing γδ cells. We suspect that the balance between the peripheral pools of IFN-γ– versus IL-17–producing γδ T cells may be substantially influenced by the maturation of $CD70^+$ dendritic cells (24) for $γδ^{27+}$ cell stimulation and by the activation of monocytes/macrophages that provide IL-1β and IL-23 for $γδ^{27-}$ cell expansion.

A general value in understanding these differential pathways of cell activation is implicit in the longstanding finding that previously activated $CD4^+$ $TCRαβ^+$ helper cell subsets can be reactivated by cytokines, including those of the IL-1 family, in the absence of TCR activation (29). However, it is important to note that our study does not exclude the TCR as being a powerful regulator of IL-17–producing γδ cells in certain scenarios (currently under examination). Rather, this study firmly establishes that during infection by various agents in vivo, different effector subsets of T cells are substantively and differentially regulated by innate and adaptive pathways. Those subsets have both direct and indirect effects on immune responses. For example, their rapid provision of IFN-γ or IL-17 can significantly impact de novo Th1/Th17 differentiation of $CD4^+$ T cells, as has been reported in both infectious (6) and autoimmune (10, 12) contexts. Thus, lymphoid stress surveillance mediated by γδ T cells may be an important component of the priming of Ag-specific immunity (2), a concept that should be further developed in both mice and humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

LN	lymph node

MuHV-4	murid herpesvirus-4
NS	nonstimulated
PAM	$Pam_3CysSerLys_4$
PbA	*Plasmodium berghei* ANKA
PEC	peritoneal cavity
sCD70	soluble rCD70
WT	wild-type

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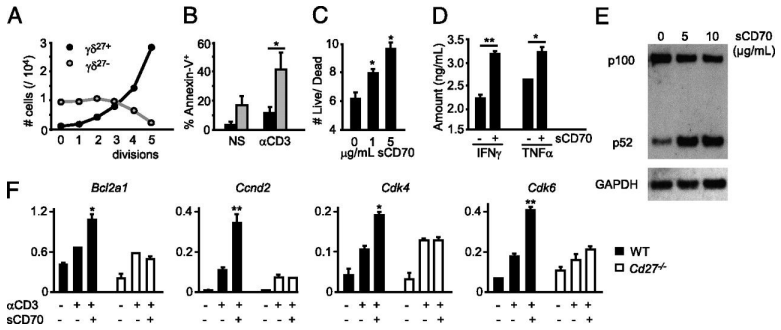

FIGURE 1.

CD27 costimulation provides antiapoptotic and proliferative signals to γδ T cells. Peripheral $\gamma\delta^{27+}$ (black filling) and $\gamma\delta^{27-}$ (gray filling) cells (*A*, *B*) or total γδ cells (*C*, *D*) were FACS-sorted from pooled spleen and LNs of C57BL/6 mice, stained with CFSE, cultured with allophycocyanin without (nonstimulated [NS]) or with anti-CD3ε mAb (αCD3) for 72 h, and then stained with Annexin V. *A*, Absolute numbers per cell division calculated based on CFSE dilution kinetics. *B*, Percentages of Annexin V^+ (apoptotic) cells. *C*, Ratio of Annexin V^- (live)/Annexin V^+ (dead) cells among divided ($CFSE^{lo}$) cells when sCD70 was added to the cultures. *D*, Cytokine bead array analysis for IFN-γ and TNF-α in the cultures' supernatants, with or without sCD70. *E*, FACS-sorted $\gamma\delta^{27+}$ cells were cultured for 16 h with anti-CD3 (1 µg/ml) and 5–10 µg/ml sCD70 or 10 µg/ml human IgG1 control. Immunoblotting analyses were performed on total cellular extracts with indicated Abs. *F*, γδ cells, FACS-sorted from $Cd27^{+/+}$ or $Cd27^{-/-}$ mice, were cultured for 6 h with or without anti-CD3 (1 µg/ml, plate-bound) and sCD70 (5 µg/ml). Quantitative real-time PCR data for *Bcl2a1*, *Cyclin D2*, *CDK4*, and *CDK6* in arbitrary units normalized to the housekeeping gene *Efa1*. Significant increases relative to anti-CD3 samples are indicated. Data in *A*–*F* are representative of three independent experiments (each involving four to six animals) with consistent results. Error bars represent SD ($n = 3$). *$p < 0.05$; **$p < 0.01$.

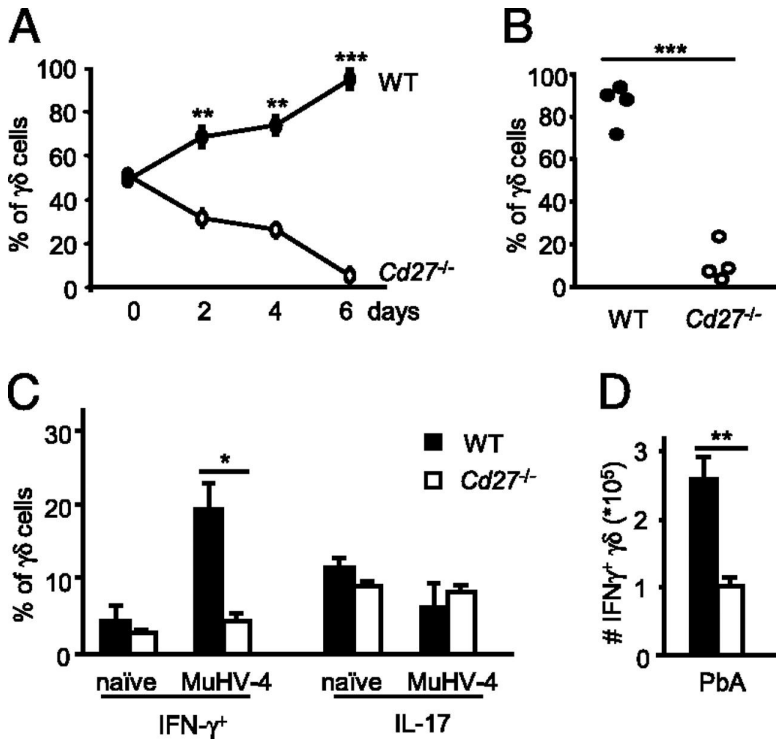

FIGURE 2.

CD27 signals are required for the expansion of IFN-γ–producing $\gamma\delta^{27+}$ cells. *A* and *B*, Total γδ cells were FACS-sorted from pooled spleen and LN of $Cd27^{+/+}$.Thy1.1 (WT) and $Cd27^{-/-}$.Thy1.2 mice. *A*, Cells were cocultured at a 50:50 ratio, stimulated with anti-CD3 in the presence of allophycocyanin, and stained for Thy1.2 at indicated time points. *B*, Cells were coinjected at a 50:50 ratio into $TCRd^{-/-}$ mice, and after 5 d, splenocytes were harvested and stained for CD3ε, TCRδ, and Thy1.2 to discriminate WT and $Cd27^{-/-}$ γδ cells. Each dot represents one recipient mouse ($n = 4$). *C*, WT and $Cd27^{-/-}$ mice were infected with MuHV-4 i.p., and cells were harvested from the PEC after 8 d. Percentage of IFN-γ^+ or IL-17^+ cells upon intracellular cytokine staining of total γδ cells, FACS-purified from PEC of WT or $Cd27^{-/-}$ mice. *D*, WT and $Cd27^{-/-}$ mice were immunized i.v. with *P. berghei* ANKA (PbA)-infected RBCs. Absolute numbers of IFN-γ^+ γδ cells in the spleen at day 4 postinfection. Data in *A*–*D* are representative of three independent experiments with similar results. Error bars represent SD ($n = 3$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

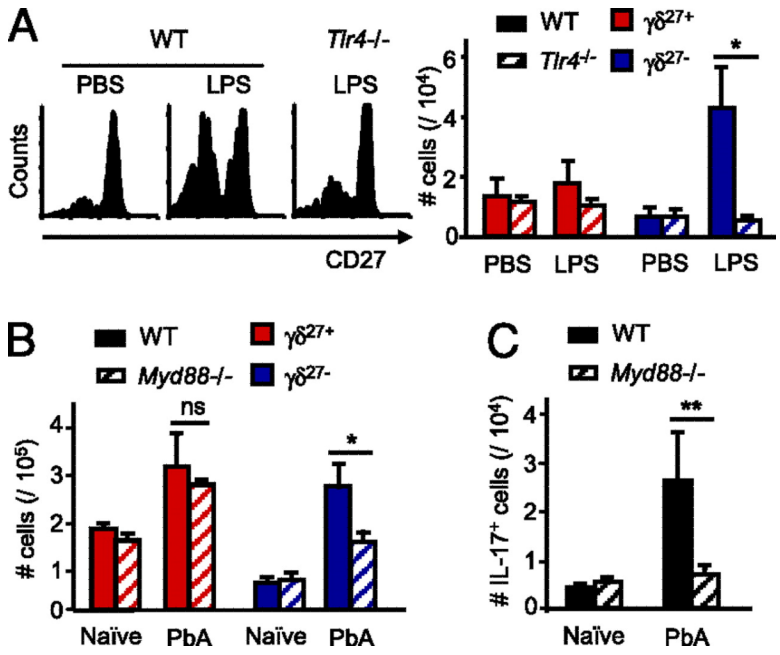

FIGURE 3.

TLR/MyD88-dependent innate signals control the pool size of IL-17–producing $\gamma\delta^{27-}$ cells in vivo. *A*, WT and *Tlr4*$^{-/-}$ mice were injected i.p. with 50 μg LPS or PBS as control and sacrificed after 3 d. CD27 expression in pregated γδ cells (*left panel*) and absolute numbers of $\gamma\delta^{27+}$ or $\gamma\delta^{27-}$ cells (*right panel*) isolated from PEC. *B* and *C*, C57BL/6 (WT) and *Myd88*$^{-/-}$ mice were immunized i.v. with *P. berghei* ANKA (PbA)-infected RBCs. Absolute numbers of $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ cells (*B*) and absolute numbers of IL-17^{+} γδ cells (*C*) in the spleen at day 3 postinfection. Data in *A*–*C* are representative of two independent experiments (each involving three animals) with similar results. Error bars represent SD (n = 3). ns, $p \geq 0.05$; *$p < 0.05$; **$p < 0.01$.

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