Reserpine improves *Enterobacteriaceae* resistance in chicken intestine via neuro-immunometabolic signaling and MEK1/2 activation

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*Salmonella enterica* persist in the chicken gut by suppressing inflammatory responses via expansion of intestinal regulatory T cells (Tregs). In humans, T cell activation is controlled by neurochemical signaling in Tregs; however, whether similar neuroimmunological signaling occurs in chickens is currently unknown. In this study, we explore the role of the neuroimmunological axis in intestinal *Salmonella* resistance using the drug reserpine, which disrupts intracellular storage of catecholamines like norepinephrine. Following reserpine treatment, norepinephrine release was increased in both ceca explant media and Tregs. Similarly, *Salmonella* killing was greater in reserpine-treated explants, and oral reserpine treatment reduced the level of intestinal *Salmonella Typhimurium* and other *Enterobacteriaceae* in vivo. These antimicrobial responses were linked to an increase in antimicrobial peptide and IL-2 gene expression as well as a decrease in CTLA-4 gene expression. Globally, reserpine treatment led to phosphorylative changes in epidermal growth factor receptor (EGFR), mammalian target of rapamycin (mTOR), and the mitogen-associated protein kinase 2 (MEK2). Exogenous norepinephrine treatment alone increased *Salmonella* resistance, and reserpine-induced antimicrobial responses were blocked using beta-adrenergic receptor inhibitors, suggesting norepinephrine signaling is crucial in this mechanism. Furthermore, EGF treatment reversed reserpine-induced antimicrobial responses, whereas mTOR inhibition increased antimicrobial activities, confirming the roles of metabolic signaling in these responses. Finally, MEK1/2 inhibition suppressed reserpine, norepinephrine, and mTOR-induced antimicrobial responses. Overall, this study demonstrates a central role for MEK1/2 activity in reserpine induced neuro-immunometabolic signaling and subsequent antimicrobial responses in the chicken intestine, providing a means of reducing bacterial colonization in chickens to improve food safety.
Results

Reserpine treatment induces norepinephrine release from intestinal cells. In an intestinal explant model (Supplementary Fig. 1), we demonstrated neurochemical release in ceca tissues at 1 h post-reserpine treatment (1 μM) using ultra-high-performance liquid chromatography (UHPLC). Culture media from reserpine-treated explants had increased levels of norepinephrine and no changes in serotonergic metabolites compared to controls (Fig. 1a). However, this norepinephrine release did not induce inflammatory damage in the explants, as pathological scores were statistically identical between groups (Supplementary Fig. 2a). Using flow cytometry to sort lymphocyte populations (Fig. 1b) potentially responsible for norepinephrine release in the ceca, Tregs (i.e., CD4+CD25+), and reserpine treatment reduced intracellular norepinephrine levels in Tregs alone (Fig. 1c). However, intracellular stores of serotonergic metabolites were unaffected by reserpine treatment (Supplementary Fig. 2b, c).

Reserpine treatment increases Salmonella resistance in ex vivo and in vivo conditions. In ceca explants from 21-day-old birds, supernatant from the reserpine-treated group had higher killing ability against Salmonella compared to that of control explants regardless of strains tested, e.g., Salmonella Typhimurium and Salmonella Kentucky (Fig. 1d). However, reserpine itself was not bactericidal (Supplementary Fig. 2d), confirming that Salmonella killing was mediated by host factors. To test in vivo reserpine-induced antimicrobial responses, we orally treated chickens with 0, 0.5, or 5 mg reserpine/kg body weight from 1 to 3 days post-hatch (dph). Reserpine treatment at either concentration did not affect the chicken weight gain at pre- (Supplementary Fig. 3a) nor post-Salmonella challenge (Supplementary Fig. 3b), nor did oral reserpine treatment induce the significant release of any neurochemicals systemically (Supplementary Fig. 3c). Given that reserpine induced antimicrobial responses ex vivo, we predicted reserpine may affect the commensal gut microbiota. However, 16S rRNA sequencing showed that reserpine treatment did not affect the levels of the majority of commensal bacteria in the ceca (Fig. 2a and Supplementary Figs. 4, 5). Nevertheless, antimicrobial responses were clearly observed after birds were challenged with Salmonella Typhimurium UK-1. At two days post-Salmonella challenge, fecal shedding of total Enterobacteriaceae and Salmonella was significantly reduced by reserpine treatment regardless of concentration (Fig. 2b). Similarly, total Enterobacteriaceae and Salmonella CFUs in ceca content were reduced by reserpine treatment at four days post-challenge (Fig. 2c). In addition to colonizing the chicken intestine, broad host Salmonella strains like UK-1 have the capacity to invade internal organs in young birds. Here, Salmonella Typhimurium UK-1 was detected in ceca, spleen, and bursa but not in the liver of challenged birds. Although Salmonella levels were statistically identical between groups in the bursa, reserpine treatment significantly reduced Salmonella levels in the spleen (Supplementary Fig. 6). Furthermore, reserpine treatment did not induce pathological inflammation at any concentration in the small intestine nor ceca (Supplementary Fig. 7), and ceca goblet cell numbers were significantly increased by reserpine treatment (Fig. 3). This is in line with a previous study demonstrating that, in mammals, reserpine treatment increases the production of intestinal mucus, which is synthesized by goblet cells in the epithelium.

Reserpine treatment increases antimicrobial peptide expression while decreasing CTLA-4 expression. To determine underlying mechanisms responsible for improved antimicrobial responses upon reserpine treatment, we measured genes expression through transcriptional changes via RT-qPCR. Expression of the regulatory cytokine IL-10, was unchanged (Fig. 4a); however, the expression of CTLA-4, a surface-bound protein associated with Tregs that downregulates immune responses, was downregulated in reserpine-treated explants versus controls (Fig. 4A). In line with this downregulated immunosuppressive factor, reserpine treatment increased gene expression of antimicrobial peptides (AMPs) like beta defensin 12 (BD-12), BD-14, and fowlcidein 1 (Fowl-1) versus controls (Fig. 4a). Furthermore, the expression of IL-2, a cytokine released by activated T cells, was also increased by reserpine treatment versus control (Fig. 4a).

Reserpine-treated explants undergo large immunometabolic shifts. To determine what global immunometabolic pathways were affected by reserpine, we used a chicken-specific kinome peptide array, which measures changes in phosphorylation activities within several signaling pathways. Overall, reserpine
Reserpine treatment altered several immunological and metabolic pathways (Table 1). In total, 414 proteins from the top 25 KEGG pathways were differentially phosphorylated upon reserpine treatment (Table 1). Within these pathways, several were involved in the EGFR signaling pathway and T cell receptor (TCR) signaling pathway, and these pathways were further analyzed. EGFR was dephosphorylated at the Tyr869 residue (Table 2). Furthermore, in the EGFR signaling pathway, mTOR was phosphorylated at Ser2448 and Thr2446 but was dephosphorylated at Ser2481 (Table 2). Uniquely, mitogen-activated protein (MAP) kinase 2 (MEK2), a component of the MEK1/2 signaling pathway, was phosphorylated at the Ser306 residue (Table 2), important for MEK2 activation. MEK2 is also involved in the TCR signaling pathway, in which CD28, a T cell co-receptor crucial for T cell activation, was phosphorylated (Table 2).

Reserpine-induced antimicrobial responses are dependent on norepinephrine and metabolic signaling. Given that reserpine treatment (1) increased intracellular norepinephrine release and (2) induced changes in EGFR and mTOR signaling pathways, we investigated the roles of these pathways in antimicrobial responses. Explants treated with norepinephrine alone similarly induced antibacterial responses in a dose-dependent manner (Fig. 4b), which was blocked by inhibiting beta-adrenergic receptors 2 and 3 (Fig. 4c). Treatment of explants with recombinant EGF alone prevented reserpine-induced antimicrobial responses (Fig. 4d). However, treatment with EGFR inhibitor AG1478 alone did not trigger any antimicrobial responses (Fig. 4d). Additionally, treatment of explants with rapamycin, an inhibitor of the mTOR pathway, increased bactericidal responses (Fig. 4e). Overall, these findings demonstrate that reserpine treatment induces antimicrobial responses through multiple signaling pathways.

MEK1/2 signaling plays a central role in reserpine-induced antimicrobial responses. In our kinome analyses, we found that these immunometabolic signaling changes were associated with MEK2 phosphorylation, suggesting MEK1/2 signaling plays a vital role in these responses. Using the MEK1/2 signaling inhibitor U0126, MEK1/2 signaling inhibition reversed the antimicrobial response induced by reserpine (Fig. 4d). Similarly, antimicrobial responses in rapamycin-treated explants were partially reversed upon MEK1/2 inhibition (Fig. 4e). Finally, antimicrobial responses in norepinephrine-treated explants were reversed upon MEK1/2 inhibition (Fig. 4f). Overall, these data demonstrate a central role for MEK1/2 signaling in the antimicrobial response induced by reserpine and other immunometabolic signaling pathways.

Discussion

Chicken products like meat and eggs are primary vehicles for salmonellosis. Reducing Salmonella colonization in the chicken...
Fig. 2 Effect of oral reserpine treatment on commensal and pathogenic bacteria. Oral reserpine treatment did not dramatically affect the composition of the commensal ceca microbiome at the genera level (a). However, post-Salmonella Typhimurium UK-1 challenge, reserpine treatment reduced total Enterobacteriaceae and S. Typhimurium UK-1 (b, c). Significant differences indicated by asterisks: *P < 0.05; **P < 0.01; ***P < 0.001. Error bars indicate the standard deviation above and below the mean.
Fig. 3 Reserpine treatment increases goblet cell numbers in the chicken ceca. Representative images of Alcian blue staining in ceca tissues (a). Total calculations of goblet cells/villus edge length (mm) (b). Scale is indicated by white bar (bottom right corner per image; 50 µm). Significant differences indicated by asterisks: **P < 0.01; ***P < 0.001. Error bars indicate the standard deviation above and below the mean.

Fig. 4 Reserpine treatment increased antimicrobial peptide (AMP) gene expression, and reserpine-induced antibacterial responses were dependent on mTOR, EGFR, and MEK1/2 signaling. AMP and IL-2 gene expression was increased by reserpine treatment while CTLA-4 gene expression was decreased (a). Norepinephrine treatment alone increased anti-Salmonella responses in explants (b), and the effect of reserpine was blocked using beta-adrenergic receptor inhibitors ICI-118551 (β2) or L-748337 (β3) (c). Reserpine-induced antibacterial activities were inhibited by MEK1/2 kinase inactivation and EGF treatment (d), and rapamycin-induced bactericidal responses are partially dependent on MEK1/2 signaling (e). Finally, norepinephrine-induced bactericidal responses are dependent on MEK1/2 signaling (f). Significant differences indicated by asterisks: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Error bars indicate the standard deviation above and below the mean.
intestine is paramount to mitigating salmonellosis in humans. In this study, we demonstrate that reserpine treatment releases intracellular stores of norepinephrine and induces significant changes in chicken ceca immunometabolism, resulting in increased antibacterial responses against *Salmonella*. The ex vivo explant model used in this study allows for preserving the totality of intestinal cell populations present in vivo while maintaining explant model used in this study allows for preserving the totality of intestinal cell populations present in vivo while maintaining the adaptive immune system. This is supported by our findings that reserpine-induced c-FOS activation may be driving these antimicrobial responses, although this remains to be determined.

This reserpine-driven increase in AMP production was associated with increased IL-2 expression and reduced CTLA-4 expression. Upon activation of naïve T cells, IL-2 production is increased, which induces further T cell proliferation, promotes CD4+ differentiation, and facilitates effector and memory CD8+ T cell formation. This activation process is dependent on the interaction between costimulatory ligand CD28, expressed on naïve T cells, and CD80/86, expressed on antigen-presenting cells (APCs). However, Tregs can interfere with this interaction via CTLA-4, which outcompetes CD28 for CD80/86 binding, inhibiting IL-2 accumulation and thus preventing T cell activation. One of the mechanisms in which *Salmonella* persists in the chicken gut is by increasing intestinal Tregs, which prevents the inflammatory responses necessary to clear *Salmonella*. Thus, we hypothesized that reserpine treatment could inactivate chicken Tregs as shown in human Tregs, which would convert anti-*Salmonella* responses in the gut. As expected, reserpine decreased CTLA-4 expression, which is constitutively expressed on Tregs. We found that CD28 was phosphorylated in reserpine-treated explants, suggesting that CD28 activation and IL-2 production were occurring due to reduced CTLA-4 levels. Furthermore, NFATC1 (but not NFATC2) was phosphorylated upon reserpine treatment. Activation of these transcription factors has been linked to IL-2 production in memory CD4+ T cells, suggesting that reserpine is increasing IL-2 gene expression through NFATC1 activation.

One notable observation is that reserpine treatment in vivo did not dramatically change the resident gut microbiota in young birds. The gut microbiota is crucial to proper animal development, driving immune and physiological maturation. Antibiotic treatment in young animals causes dramatic changes in their gut microbiota, which can predispose them to bacterial infection and physiological dysfunction later in life by depleting populations of gut microbes crucial for normal development. Thus, oral reserpine treatment in day-old birds is a feasible way to promote resistance against *Salmonella* without negatively affecting the developing gut microbiota, although the long-term effects of early-age reserpine treatment on the gut microbiota are unclear. Although changes in Fusobacteria, *Lactobacillaceae*, and *Erysipelotrichaceae* were induced by reserpine treatment, these changes were not consistent between reserpine-treated groups and did not appear to be associated with any biological parameter measured in this study (antimicrobial responses, inflammation, mucus production, etc.). Thus, the biological impact of these specific shifts in the commensal microbiota is unclear and does not contribute to the host responses investigated in this study. Still, this lack of antimicrobial activity may appear to contrast the reserpine-induced antimicrobial responses seen in our ex vivo explant model. In birds, innately produced gallinaceins are the primary AMPs produced in the intestine at post-hatch, peaking at days 1–3 post-hatch and begin to drop by day 4 post-hatch, in which AMPs controlled through intestinal Tregs, which coincidentally migrate from the thymus to the chicken intestine around day four post-hatch. Dhawan and colleagues (2016) determined that specific subsets of inflammatory responses. Given these findings were reflected in our study, reserpine-induced c-FOS activation may be driving these antimicrobial responses, although this remains to be determined.

### Table 1 Top 25 KEGG pathways in reserpine-treated explants compared to non-treated controls.

| KEGG pathway                  | Observed protein count | False discovery rate |
|-------------------------------|------------------------|----------------------|
| MAPK signaling                | 54                     | 2.00 × 10^{-35}      |
| Insulin signaling             | 41                     | 3.14 × 10^{-34}      |
| Pathways in cancer            | 63                     | 7.96 × 10^{-33}      |
| PI3K-Akt signaling            | 51                     | 1.22 × 10^{-29}      |
| ErbB signaling                | 29                     | 2.58 × 10^{-27}      |
| EGFR signaling pathway        | 29                     | 2.58 × 10^{-27}      |
| Neurotrophin signaling        | 32                     | 8.41 × 10^{-26}      |
| Focal adhesion                | 38                     | 8.41 × 10^{-26}      |
| AMPK signaling                | 32                     | 1.55 × 10^{-25}      |
| MicroRNAs in cancer           | 34                     | 2.76 × 10^{-25}      |
| Central carbon                | 26                     | 1.69 × 10^{-24}      |
| Metabolism in cancer          | 29                     | 3.38 × 10^{-24}      |
| T cell receptor signaling     | 29                     | 3.38 × 10^{-24}      |
| Proteoglycans in cancer       | 35                     | 4.09 × 10^{-23}      |
| Insulin resistance            | 28                     | 2.62 × 10^{-22}      |
| Ras signaling                 | 35                     | 3.65 × 10^{-21}      |
| HIF-1 signaling pathway       | 24                     | 1.22 × 10^{-18}      |
| Autophagy-animal              | 26                     | 1.22 × 10^{-18}      |
| Regulator of actin cytoskeleton | 31                    | 1.22 × 10^{-18}      |
| Hepatitis C                   | 26                     | 2.93 × 10^{-18}      |
| FoxO signaling                | 25                     | 2.61 × 10^{-17}      |
| Chemokine signaling           | 28                     | 3.59 × 10^{-17}      |
| Toll-like receptor signaling  | 22                     | 2.97 × 10^{-16}      |
| mTOR signaling                | 25                     | 3.18 × 10^{-16}      |
| Adipocytokine signaling       | 19                     | 8.75 × 10^{-16}      |
| B cell signaling              | 19                     | 1.29 × 10^{-15}      |

The rows in bold indicate the immune or metabolic pathways focused on in this study.
Table 2 Phosphorylation status of proteins in the T cell receptor and epidermal growth factor signaling pathways in ceca explants treated with reserpine.

| Peptide | Uniprot accession | Phosphorylation site | Fold change | p-value |
|---------|------------------|----------------------|-------------|---------|
| **T cell receptor signaling pathway** | | | | |
| PLCG2  | P19174           | Y783                 | −1.470      | 0.00001 |
| RAFl   | P04049           | S338/S259            | 1.2419/1.08925 | 0.00001/0.00016 |
| **MEK2** | **P36507**     | **S306/S222**        | **1.510/1.323** | **0/0.0003** |
| MAP3K8 (TPL2) | P41279        | S400/T290            | −1.471/−1.26824 | 0/0.00059 |
| AKT3   | Q9Y243           | T305                 | −1.839      | 0       |
| ZAP70  | P43403           | Y319                 | −1.510      | 0       |
| PAK1   | Q13153           | T423                 | 1.236       | 0.0003  |
| NFATC3 | Q12968           | S241                 | −1.431      | 0.0003  |
| c-FOS  | P10747           | S444                 | 1.195       | 0.0001  |
| CD28   | P17047           | S191                 | 1.286       | 0.00121 |
| LCK    | P06239           | Y505                 | 1.116       | 0.0003  |
| PDPK1  | O15530           | S241                 | 1.283       | 0.0003  |
| TAK1   | P03318           | S439                 | 1.277       | 0.006   |
| IKK-B  | O15111           | S180                 | −1.247      | 0       |
| JUN    | P05412           | S63/S73              | −1.447/−1.734 | 0/0     |
| **GRB2** | **P62993**      | **Y209**             | **1.757/1.169** | **0/0.00161** |
| **NFATC1** | **P0121**     | **S269/S245**        | **1.195**   | **0.00098** |
| **h-RAS** | **P00575**     | **S167**             | **1.234**   | **0.001** |
| **NF-kB p105** | **P19838** | **S137/S932**        | **−1.147/−1.141** | **0.007/0.009** |
| **IL6R** | **P40189**      | **S782/Y915**        | **−1.169/1.259** | **0.0142/0.00005** |
| **IL7R** | **P16871**      | **Y449**             | **−1.177**  | **0.0335** |
| **IL23R** | **P29460**     | **Y209**             | **1.390**   | **0.001** |
| SOCS   | P01100           | S338/S259            | 1.242/2.089 | 0.0006/0.01 |
| **PDGFRA** | **P16234**    | **Y1018/Y720**       | **−2.174/−1.135** | **0.00006/0.00006** |
| **PDGFRB** | **P00619**     | **Y579/Y751**        | **−1.141/−1.127** | **0.002/0.002** |
| JAK2   | O60674           | Y966/Y1007           | 1.226/−1.260 | 0.002/0.007 |
| JAK1   | P23458           | Y993/Y1034           | −1.384/−1.174 | 0.015/0.016 |
| **STAT1** | **P42224**     | **Y701**             | **−1.277**  | **0** |
| **STAT4** | **P42228**     | **S722**             | **−1.338**  | **0.002** |
| **STAT3** | **P40763**     | **S727**             | **−1.302**  | **0.0004** |
| **Epidermal growth factor receptor signaling pathway** | | | | |
| **RPS6KB1** | **P23445**    | **T412**             | **1.256**   | **0.0003** |
| PLCG1  | P19174           | Y783                 | −1.97       | 0.0001  |
| RAFl   | P04049           | S338/S258            | 1.242/2.089 | 0.0006/0.001 |
| **PDGFR** | **P16234**     | **Y1018/Y720**       | **−2.174/−1.135** | **0.00001/0.0001** |
| PDGFRB | P00619           | Y579/Y751            | **−1.141/−1.127** | **0/0.008** |
| **MEK2** | **P36507**     | **S306/S222**        | **1.51/1.327** | **0/0.03** |
| AKT3   | Q9Y243           | T305                 | −1.839      | 0       |
| KDR    | P35968           | Y1214                | −1.496      | 0       |
| STAT3  | P40763           | S727                 | −1.302      | 0.0004  |
| **EGFR** | **P00533**     | **Y869**             | **−1.242**  | **0.007** |
| **BRAF** | **P10174**     | **Y783**             | **−1.97**   | **0.0001** |
| **PRKCB** | **P04049**     | **S338/S258**        | 1.242/2.089 | 0.0006/0.001 |
| **MET** (HGFR) | **P0121**     | **S137/S932**        | **−1.147/−1.141** | **0.007/0.009** |
| GSK3B  | P49841           | S389                 | 1.161       | 0.005   |
| **FGFR3** | **P22607**     | **Y760/Y724**        | **−1.258/−1.194** | **0.015/0.016** |
| EIF4ER1 | P13541          | T37                  | 1.16       | 0.04    |
| JAK1   | P23458           | Y993/Y1034           | −1.384/−1.174 | 0.002/0.002 |
| **mTOR** | **P42345**     | **S2448/S2246**      | **1.721/1.41/1.672** | **0/0.00001/0.0006** |
| RPTOR  | P42345           | S863                 | 1.245       | 0.00025 |
| PTEN   | P42345           | S380/Y240            | −1.14/1.247 | 0.025/0.002 |
| SRC    | P10174           | S17                  | 1.154       | 0.004   |
| SHC3   | P42345           | Y427                 | 1.208       | 0.02    |
| JAK2   | P42345           | Y966/Y1007           | 1.226/−1.260 | 0.0006/0.0006 |
| GRB2   | P42345           | Y209                 | 1.390       | 0.001   |
| SHC1   | P42345           | Y262                 | −1.208      | 0.001   |
| HRAS   | P42345           | T35                  | −1.234      | 0.001   |
| PRKCA  | P42345           | S657/T638            | −1.135/−1.204 | 0.005/0.03 |
| FGFR2  | P42345           | S782                 | −1.190      | 0.02    |

The phosphorylation status of each significant protein in ceca explant after treatment with reserpine was determined by entering the respective Uniprot accession into phosphorylation site, finding the annotation of the site of interest, and accounting for the phosphorylation fold change (increased or decreased) of that site. Uniprot IDs and phosphorylation sites listed are human orthologs of chicken peptides. Bolded peptides indicate targets of interest in this study.
intestinal Tregs are crucial for regulating AMP responses, although the subset of Tregs responsible for this mechanism in chickens is still unclear and warrant further investigation. In humans, reserpine inhibits intracellular vesicle storage of catecholamines such as norepinephrine, which induce autocrine/paracrine signaling loops that suppress Treg function and stimulate immune activation. In this study on chickens, reserpine treatment increased norepinephrine release from both explants and intestinal Tregs. Thus, Tregs at least partially contribute to the total pool of norepinephrine released by intestinal cells. However, in our hands and due to limited reagents and methods for primary chicken cell cultures, we could not culture chicken intestinal Tregs for longer than six hours, preventing any direct examination of reserpine on Treg immunosuppressive function. However, we did find that treatment with norepinephrine alone at the physiological concentration released after one hour of reserpine treatment could stimulate antibacterial responses, which was dependent on beta-adrenergic receptors. Norepinephrine is a well-known mediator of neuroimmunological responses, inducing cytokine production, cell proliferation, and antibiotic activity by lymphocytes. Rapamycin, originally derived from the soil bacterium Streptomyces hygroscopicus, is commonly used as an mTOR inhibitor. In this study, reserpine induced differential mTOR phosphorylation at multiple sites upon reserpine treatment. Phosphorylation of S2448 and T2446 is carried out by the kinase S6K and pS2448 drives mTORC1 activation. In this study, mTORC1 may have been activated upon reserpine treatment, as these two mTORC1 sites, S6K, and raptor (i.e., RPTOR) were all phosphorylated. However, mTORC1 activation does not play a role in these antimicrobial responses, as activating mTOR via rapamycin treatment induced similar antimicrobial responses as reserpine treatment. Although these mTOR sites were phosphorylated, S2481 was uniquely dephosphorylated upon reserpine treatment. The sole site for mTOR autophosphorylation and rapamycin treatment induced similar antimicrobial responses as reserpine treatment. However, we did find that treatment with norepinephrine alone at the physiological concentration released after one hour of reserpine treatment could stimulate antibacterial responses, which was dependent on beta-adrenergic receptors. Norepinephrine is a well-known mediator of neuroimmunological responses, inducing cytokine production, cell proliferation, and antibiotic activity by lymphocytes. Rapamycin, originally derived from the soil bacterium Streptomyces hygroscopicus, is commonly used as an mTOR inhibitor. 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Intestinal lymphocyte extraction and flow cytometry. Cells were extracted from the chicken lamina propria as previously described. To sort for specific T cell populations (CD4+ or CD8+), cells were resuspended in Zimbait violet dye (1:100 solution) and incubated for 20 min at room temperature in the dark. Cells were then centrifuged at 300 × g for 5 min at room temperature, and pellets were resuspended via sorting buffer (PBS with 1% FBS) and incubated for 10 min at 4 °C for a blocking step. Thereafter, cells were centrifuged for 5 min at 300 × g, and then resuspended with 10 µg/ml anti-CD4 and 10 µg/ml anti-CD25 manually conjugated with Alexa-555 or Alexa-488 fluorophores, respectively. Following a 30 min incubation in the dark at 4 °C, cells were then washed with sorting buffer and viable CD4+CD25+ and CD4−CD25− populations were sorted via FACS Aria III (BD Biosciences) at Iowa State University’s core facility. The gating strategy is exemplified in Supplementary File 1.

Bacterial assays against Salmonella. Following explant incubation, media from individual explants were centrifuged at 12,000 × g for 30 min at 4 °C, and the supernatant was stored at −80 °C until ready for use. S. enterica strains (Supplementary Table 1) were grown overnight on LB agar (0.1% glucose), and individual colonies were added to PBS until OD600 = 0.1. This inoculum was subsequently diluted in PBS until 10^2 CFU/100 µl/mL. Explant supernatants were added to Salmonella innoculum at 1:1 ratio and incubated for six hours at 39.5 °C. Solutions were then serially diluted and plated on MacConkey for bacterial enumeration. All bacterial assays were run in triplicate.

In vivo reserpine treatment and Salmonella challenge. One-day-old white leghorn chicks (Valo BioMedical, IA) were orally treated daily with 0.5, 0.5 mg reserpine per kg body weight (100 µl) for three days. At four days old, chicks were orally inoculated with 10^9 (100 µl) CFU Salmonella Typhimurium strain UK-1 (Supplementary Table 1). Prior to reserpine treatment and Salmonella challenge, birds were fasted from food and water for at least 4 h, and food and water were returned to pens 30 min post-inoculation and challenge, respectively. Two days post-challenge, ceca were serially diluted and plated onto MacConkey agar for Enterobacteriaceae and Salmonella enumeration. Four days post-challenge, ceca contents, spleen, liver, and bursa were collected from each bird, homogenized, and plated onto MacConkey agar. Chicken weights were collected daily throughout the study.

DNA isolation and 16S rRNA sequencing. Total DNA was isolated from ceca contents (homogenized from both cecal loops per bird; n = 9 or 10 per treatment group) using the DNeasy PowerLyzer PowerSoil Kit (Qiagen). Extracted DNAs were determined for quality via NanoDrop 2000 spectrophotometer (A260/A280). Concentrations were then determined via Qubit dsDNA standard range kit (Thermo Fisher Scientific). DNAs were used for library preparation using the MiSeq and HiSeq500 kit ( Illumina) following all the manufacturer’s instructions with 151 × 151 paired-end MiSeq sequencing (Illumina). 16S rRNA sequencing was performed at the Iowa State DNA facility using Illumina MiSeq (v3). For sequence analysis, using the QiIME2 (version 2019.10) pipeline, sequences were demulti-plexed using the demux emp-paired function and denoised using the plugin DADA2. SILVA database at the 99% operational taxonomic units (OTUs) span of the V4-V5 16S rRNA region (806R: CAGCAAGGAGACCGGTACGAGAATAGTCAAGCCACGGGACTACNVGGGTWTCAATTA; 515F: ATGTACGAGCGACCACGGCAAATGCGTACGACGCTXXXXXXXGTTGAATGTTGTCYGACGACCCGCGGTGAA) was used to classify each of the reads using QiIME2’s featureclassifier function. For more details, please refer to the GitHub repository at USagenomics/2021_Aug_MelhaMellata_reserpine_study (github.com). The 16S rRNA dataset is available in the NCBI Sequence Read Archive (SRA) repository with accession BioProject ID PRJNA755726.

Intestinal pathology scoring and goblet cell enumeration. Explants were placed into 4% paraformaldehyde (PFA) and stored at RT. Subsequently, 5 µm paraffin-embedded cross-sections were stained with hematoxylin and eosin (H&E) to assess gut inflammation. Parameters measuring inflammation (i.e., focal, multifocal, diffuse), infiltrate (i.e., presence of heterophils, lymphocytes, macrophages as well as hemorrhages), necrosis (i.e., focal, multifocal, diffuse), and location (i.e., lamina propria, villous lamina propria, crypt lamina propria) were used. All analyses were performed by a certified pathologist at Iowa State University. VWF expression in ceca tissue, sections were stained with Alcian blue to enumerate goblet cells. To quantify goblet cell numbers per villus edge length, the length of the intestinal epithelium was measured using computer software. Goblet cells were then individually counted and divided by villus edge length. Counting on five replicate sections was performed on bird, and 8–10 birds were analyzed per treatment group.

RT-qPCR. Total RNA was extracted from explant tissues using the PureLink RNA Mini Kit (Life Technologies), and high quality RNAs (A260/A280 ratios ≥ 2.0) were assessed via Nanodrop 2000 and quantified via Qubit 2.0 Fluorometer. Reverse transcription assays were performed via High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) to attain cDNA. Thereafter, SYBR Green (Thermo Scientific) three-step cycling qPCR reactions were performed on StepOnePlus for individual genes (Supplementary Table 2) for 45 cycles. Differences in gene expression were assessed via 2−ΔΔCt method using the housekeeping gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control.

Chicken-specific immunometabolic kinase peptide array. Following incubation, ceca explants were flash-frozen, stored at −80 °C, and transported overnight on dry ice to the University of Delaware. Peptide array protocol and analyses were carried out as previously described. The resulting data output was then used in downstream applications such as STRING and KEGG databases used to pinpoint changes in the protein–protein interactions and signal transduction pathways.

Statistics and reproducibility. Statistical comparisons for UHPLC and Salmonella resistance data were performed via Student’s t-test or one-way ANOVA on GraphPad Prism software. For the kinome array, signal intensities from scanned array images were arranged into the PIKKA2 input format in Excel, and resultant data were subsequently analyzed via PIKKA2 peptide array analysis software (http://saphire.usask.ca/saphire/pika/index.html). After normalizing these data, we performed comparisons between reserpine-treated and untreated explants, calculating fold change (treatment/control) and a significance P-value, which was calculated by conducting a one-sided paired t-test between treatment and control values for a given peptide. The resultant fold change and significance values were used to generate optional pathway analysis via standard R statistical functions or online analysis platforms. All in vivo experiments were done in duplicate, and in vitro experiments were performed in triplicate.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The 16S rRNA dataset is available in the NCBI Sequence Read Archive (SRA) repository with accession BioProject ID PRJNA755726. Raw kinome data are provided in Supplementary Data 1.

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Author contributions
G.R. and M.M. conceived and designed the experiments, as well as wrote the manuscript. G.R., M.K., R.A., and M.M. performed the experiments, analyzed the data, and revised the manuscript. R.A., M.K., M.L., and M.M. contributed reagents, materials, and analysis tools. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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