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## Mediation of Interleukin-23 and Tumor Necrosis Factor–Driven Reactive Arthritis by Chlamydia -Infected Macrophages in SKG Mice

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## **Chlamydia-infected Macrophages mediate Interleukin-23 and Tumor Necrosis Factor-driven Reactive Arthritis in SKG Mice**

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**Running head: Chlamydia-infected Macrophages drive SKG Reactive Arthritis**

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**Abstract**

**Objective:** ZAP-70<sup>W163C</sup> BALB/c (SKG) mice develop reactive arthritis (ReA) after *Chlamydia muridarum* (Cmu) infection. Since intracellular pathogens enhance their replicative fitness in stressed host cells, we examined how myeloid cells taking up Cmu drive arthritis.

**Methods:** Female SKG, *I17a*-deficient SKG and BALB/c mice were genitally infected with Cmu or Cmu Luciferase. Cmu dissemination was assessed by *in vivo* imaging or genomic DNA amplification. Macrophages were depleted using clodronate liposomes.

Anti-TNF, anti-IL-23p19 were administered after infection or arthritis onset. *Hspa5*, *Tgtp1*, *Ii23a*, *Ii17a*, *Ii12b* and *Tnf* expression was compared in SKG and BALB/c mice.

**Results:** One-week post infection, macrophages and neutrophils infiltrated the uterus; both carried Cmu DNA to the spleen. Cmu load was higher in SKG than BALB/c. Macrophage depletion reduced Cmu load and prevented arthritis. Compared with BALB/c, expression of *Ii23a* and *Ii17a* was increased in SKG uterine and splenic neutrophils. Anti-IL-23p19 during infection or *Ii17a* deficiency suppressed arthritis. *Tnf* was over-expressed in SKG joints within one-week post infection and persisted. TNF inhibition during infection or at arthritis onset suppressed arthritis. Endoplasmic reticulum stress was constitutively increased in SKG joints but was induced, with immunity-related GTPase, by Cmu infection in uterus.

**Conclusion:** The load of Cmu is higher in SKG than BALB/c mice. While proinflammatory IL-23 produced by neutrophils contributes to the initiation of Cmu-mediated ReA, macrophage depletion reduces Cmu dissemination to other tissues, tissue burden, and arthritis. TNF inhibition suppresses arthritis. Our data suggest that enhanced bacterial dissemination in SKG macrophages drives TNF production required for persistent arthritis.

## Introduction

Chlamydia-induced reactive arthritis (ReA) belongs to the spondyloarthritis (SpA) group of diseases. ReA occurs in 4 to 15% of *Chlamydia trachomatis* infections (1). *Chlamydia* is the most common cause of ReA overall. Clinical features typically include enthesitis, peripheral arthritis, inflammation of the axial skeleton, conjunctivitis and a psoriasis-like skin rash. Symptoms develop a few weeks after infection, which can be silent, partially explaining why ReA remains under-diagnosed. The mechanisms of ReA induction and persistence are not fully understood. However, new insights were made with the discovery that ZAP70<sup>W163C</sup>-mutant BALB/c (SKG) mice develop features of human ReA five weeks after *Chlamydia muridarum* (Cmu) infection, a mouse-adapted *Chlamydia trachomatis* related pathogen (2).

The SKG ZAP70 mutation results in attenuated T cell receptor signaling, promoting a state of relative immunodeficiency. Cmu-induced ReA developed in association with deficient pathogen control by major outer membrane protein (MOMP)-specific IFN- $\gamma$  and IL-17-producing CD4<sup>+</sup> T cells and increased T cell tumor necrosis factor (TNF)

production (2). Arthritis development depended on infection with live pathogen and was pathogen dose dependent (2).

Macrophages and neutrophils are thought to play an important role in ReA, through the systemic dissemination of pro-inflammatory *Chlamydia pneumoniae* and *trachomatis* pathogen-associated inflammatory molecules (PAMPs) (3). *Chlamydia* may paralyze neutrophil pathogen control mechanisms, such as extracellular trap formation (4). Toll-like receptor (TLR) stimulated macrophages are substantial producers of IL-23 in SpA, while neutrophils produce IL-23 in inflammatory bowel disease (5-8). IL-23 is a critical cytokine for the maintenance of Th17 cells and for stimulation of other pro-inflammatory cytokines production in SpA (9). In HLA-B27<sup>+</sup> patients and HLA-B27 SpA transgenic rats, endoplasmic reticulum (ER) stress may favor macrophage and dendritic cell (DC) production of IL-23 in the presence of TLR ligands (6, 10). Furthermore, *Salmonella enterica*, a ReA-associated pathogen, was shown to exploit ER stress in HLA-B27-expressing cells to promote its own intracellular replication (11). Autophagy, a highly conserved process that recycles defective organelles and non-functional proteins and contributes to control of intracellular microorganisms, was shown to contribute to intestinal IL-23 production in ankylosing spondylitis (AS) (12). Of interest, autophagy sequesters the NF- $\kappa$ B regulatory protein A20, promoting pro-inflammatory cytokine and chemokine production (13). Interferon-inducible immunity-related GTPase (IRG) proteins are required for control of intracellular bacterial infection (14). IRG proteins are recruited into *Chlamydia* inclusions and restrict *Chlamydia* replication by routing inclusions to the lysosome and inducing autophagy (15, 16).

Given that Cmu burden and its immune control are critical for the development and persistence of arthritis in SKG mice, and that intracellular pathogens enhance their own replicative fitness in stressed host cells, we examined how SKG macrophages and neutrophils taking up Cmu contributed to development of Cmu-induced ReA.

## **Materials and Methods**

### **Mice**

Female BALB/c, SKG mice (ZAP-70<sup>w163c</sup>-mutant BALB/c mice) and IL-17<sup>-/-</sup> SKG mice aged 6-10 weeks were bred and maintained under specific pathogen-free conditions (4 to 6 mice per conventional cage) at the Universities of Queensland (Australia) and

Grenoble-Alpes (France, agreement: C 3851610006). BALB/cJRj mice were obtained from Janvier Labs. Housing condition was humidity 50%±20%, temperature 19-23°C, 12/12hrs dark/light sessions and ad libitum access to food and water. Neither SKG mice nor IL-17<sup>-/-</sup> SKG mice developed spontaneous disease under these conditions. Animal study was conducted in accordance with the European Directive 2010/63/EU and experimental procedures were approved by Ethical Committees: #7781-201611251629264v1 (French Research Ministry) and UQDI/467/12/NHMRC (University of Queensland).

### ***Chlamydia muridarum* bacteria**

The same strain of Cmu (Weiss strain) was used for all experiments, grown and purified as previously described (2). Cmu (Weiss strain) expressing luciferase and Green Fluorescent Protein (pGFP-Luc-Cmu) was engineered as previously described (17). A plasmid-deficient strain of Cmu (Nigg strain), generated by treatment with novobiocin (O'Connell and Nicks), was a generous gift from Dr Catherine O'Connell, University of North Carolina at Chapel Hill (18). Chlamydial vaginal load was determined by direct inoculation of vaginal swabs sample onto McCoy cell monolayers as previously described (2). Cmu DNA was detected using the following primers: 5' - GGAGCAAATCCTCAAAGCTG-3', 5'-ATCCCAGTCATCAGCCTCAC-3' and quantified using SYBR green technique. Cmu gene copy number per cell was calculated by dividing the Cmu gene copy number by the number of sorted cells.

### **Infection of SKG mice**

Mice aged 6 to 10 weeks were primed by a subcutaneous injection of 2.5 mg of medroxyprogesterone acetate (Depo-Provera®, Pfizer) one week before infection and then infected with 7.10<sup>3</sup> to 1.10<sup>6</sup> inclusion-forming units (IFU) of Cmu intravaginally, as previously described (2). Infected and non-infected mice were placed in different cages and manipulated separately to avoid cross-contamination.

### **Clinical and histopathologic assessment**

Paw swelling, the primary experimental outcome, was evaluated by the same observer for 12 weeks post infection (pi) by measuring the thickness of hind paws with a digital caliper and the paw inflammation index (paw swelling variation from baseline) was calculated as in (2). Joints were fixed at week 12 in 10% formalin, embedded in paraffin

and stained with hematoxylin and eosin. Two blinded and independent readers assigned a histological score of inflammation on a scale of 0-5 for joints and skin (2).

### ***In vivo* imaging**

BALB/c and SKG mice were infected intravaginally with  $2 \cdot 10^5$  IFU of pGFP-Luc-Cmu. Mice were imaged, as previously described (17) using the Xenogen IVIS imaging system (PerkinElmer, MA, USA) one week pi. Intensity of bioluminescence was analyzed by using living image software (PerkinElmer).

### **Anti-Cmu MOMP antibody measurement**

Anti-*Chlamydia* MOMP IgG and IgA were measured in serum of infected mice 12 weeks pi, using enzyme-linked immunosorbent assay (ELISA), as previously described (2).

### **Macrophage depletion**

Macrophages were depleted by intraperitoneal injection of liposomal clodronate (Liposoma, The Netherlands). Upon Cmu infection, mice received an initial clodronate dose of 2 mg per 20g of mouse body weight, followed by weekly injections of 1 mg per 20 g of mouse body weight, for 12 weeks (n=7 mice analyzed). The control group was injected with phosphate buffered saline (PBS) liposomes (n=7 mice analyzed).

### **Anti-TNF, anti-IL-23p19 and antibiotics treatment**

Mice were treated by subcutaneous injections of anti-mouse TNF antibody (2mg/kg/week of mouse-adapted certolizumab pegol antibody PEGylated Fab with 40kD Nektar PEG (CA156-00501, UCB) (n=18) or 4.5 mg/kg/3 days of etanercept (n=18) (Enbrel, Pfizer) or by intra-peritoneal injection of anti-mouse IL-23p19 antibody (60 µg/week, LSN2479016, Eli Lilly and Company, Indianapolis, USA) (n=17) from week 1 (at infection) to week 12 or from week 5 (disease onset) to week 12. The control groups were injected with PBS or isotype controls (n=8-13 mice per experiment). Rifampicin (0.4 mg/day intraperitoneally, Sanofi-Aventis) and doxycycline (0.3 mg/day by gavage, Alphapharm) were administered (n=11 mice) from week 7 through week 9 pi. Adverse events and experimental group are described in **supplementary table 1**.

### **Isolation of uterine and splenic mononuclear cells and neutrophils**



Genital tract and spleen from Cmu infected mice were collected 7 days pi for cell isolation (**Supplementary method**). Cells were used in flow cytometry or for myeloid cell isolation by Histopaque gradient technique (Sigma). Macrophages (MHCII<sup>+</sup>CD11c<sup>+</sup>CX3CR1<sup>+</sup>), dendritic cells (MHCII<sup>+</sup>CD11c<sup>+</sup>CD103<sup>+</sup>), neutrophils (MHCII<sup>-</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup>) and lymphocytes (CD3<sup>+</sup>) were sorted with a BD FACSAria Fusion Sorter (BD Biosciences) or MoFlo Astrios EQ™ Sorter (Beckman Coulter).

### Flow cytometry analysis

Cells were treated with anti-CD16/32, stained with LIVE/DEAD™ Aqua, anti-mouse CD45.2 APC (clone 104 , Biolegend), anti-mouse I-A/I-E Pacific Blue (clone M5/114.15.2, Biolegend), anti-mouse Ly6G PerCP-Cy5.5 (clone 1A8, Biolegend), anti-mouse CD11b AF700 (clone M1/70, Biolegend), anti-mouse CD11c APC/Cy7 (clone N418, Biolegend), anti-mouse CX3CR1 FITC (clone SA011F11, Biolegend), anti-mouse CD103 PE (clone 2E7, Biolegend) and anti-mouse CD3 FITC (clone 17A2, Biolegend), adapting a method identifying intestinal myeloid populations (7). Cell numbers were enumerated using Flow-Count Fluorospheres (Beckman Coulter), as previously described (19). Data were acquired on a LSRII Fortessa X20 flow cytometer (BD Biosciences) (**Supplementary method**).

### Quantitative real time PCR

Uterine and splenic macrophage/DC, neutrophils and lymphocytes from infected SKG and BALB/c mice were analyzed after total RNA was isolated using RNeasy minikit (Qiagen) and cDNA was prepared using Tetro cDNA synthesis kit (Bioline). RNA from genital tracts and hind paws were isolated 1 and 5 weeks pi respectively and cDNA was generated using SuperScript™ III First-Strand Synthesis SuperMix Kit (Invitrogen) (**Supplementary method**). Quantitative real-time PCR for *I112b* (Forward 5'-GGAAGCACGGCAGCAGAATA-3'; reverse 5'-AACTTGAGGGAGAAGTAGGAATGG-3'), *I123* (Forward 5'-GCTGTGCCTAGGAGTAGCAG-3'; reverse 5'-TGGCTGTTGTCCTTGAAGTCC-3'), *I117a* (Forward 5'-TCTCCACCGCAATGAAGACC-3'; reverse 5'-CACACCCACCAGCATCTTCT-3'), *Tnf* (Forward 5'-CATCTTCTCAAATTCGTGTGACAA-3'; reverse 5'-TGGGAGTAGACAAGGTACAACCC-3' or *Tnf*, Mm00443258\_m1, Thermofisher), *Hspa5* (Forward 5'-ACTTGGGGACCACCTATTCCT-3'; reverse 5'-GTTGCCCTGATCGTTGGCTA-3'), *Tgtp1* (Forward 5'-

TGCACAGATGGGGATGAATTTTC-3'; reverse 5'-TCACTGTCTGAGAGACTCCTGA-3), *Hprt* (Forward 5'- CCCCAAAATGGTTAAGGTTGC -3'; reverse 5'-AACAAAGTCTGGCCTGTATCC -3' or Forward 5'-TCAGTCAACGGGGGACATAAAA-3'; reverse 5'-GGGGCTGTACTGCTTAACCAG-3') and *Gapdh* (Mm99999915\_g1, ThermoFisher) was performed using SYBR green or TaqMan technology. All real-time RT-PCR data were normalized relative to a housekeeping gene (*Hprt* or *Gapdh*). Data are reported as relative fold change compared to *Hprt* calculated using the  $\Delta C_t$  method or as relative fold change in gene expression compared to non-infected BALB/c using the  $\Delta\Delta C_t$  method.

### Statistical analysis

The normality of the distribution was assessed by the Kolmogorov-Smirnov test. One-way non-parametric ANOVA (Kruskal Wallis test) and Mann-Whitney tests were used for non-normally distributed data and t-test or one and two-way ANOVA with post hoc Tukey's test for normally distributed data. *P* values < 0.05 (2-tailed) were considered significant. The number of animals needed to achieve statistical power was estimated from previous experiments: 8 mice per group with an  $\alpha$ -risk of 0.05 reach a power of 80% (2). Statistical analysis was performed using GraphPad Prism 8.4.3 (GraphPad Software, San Diego, USA).

### Results

#### Macrophages and neutrophils infiltrate the genital tract of infected mice and systemically disseminate Cmu

Cmu genital infection induces a pro-inflammatory response. By flow cytometric analysis of cellular infiltrate into the uterine horns, the number of macrophages and neutrophils, but not DCs, increased in both BALB/c and SKG mice within one-week post-Cmu genital infection (**Figure 1A-C and Supplementary Figure 1**). Previously, Cmu DNA was found in CD11b<sup>+</sup> myeloid cells in SKG mouse spleen and lymph node 1 week pi (2), indicating that myeloid cells may transport Cmu from the site of infection to the spleen. To identify which myeloid cells transport Cmu to the spleen after genital tract infection, we infected SKG mice with Cmu, and one-week later isolated splenic DCs (MHCII<sup>+</sup>CD11c<sup>+</sup>CD103<sup>+</sup>), macrophages (MHCII<sup>+</sup>CD11c<sup>+</sup>CX3CR1<sup>+</sup>), and neutrophils (MHCII<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup>). Cmu DNA was detected in macrophages and neutrophils, but not DCs, indicating that these cells take up and disseminate Cmu

within 1 week of infection (**Figure 1D**). When normalized against number of sorted cells, Cmu DNA copy number was higher in macrophages than neutrophils, and also higher in SKG infected mice compared to BALB/c (**Figure 1E**).

To track Cmu dissemination and burden after infection of the genital tract epithelium, we used Cmu expressing luciferase and GFP (pGFP-Luc-Cmu). Luciferase signal ascended from lower to upper genital tract by 7-11 days pi in SKG and BALB/c mice infected with pGFP-Luc-Cmu (**Figure 2A**). Vaginal shedding of wild type (WT) and pGFP-Luc-Cmu was significantly higher in SKG than BALB/c mice for 2 weeks pi (**Figure 2B**). To determine the contribution of macrophages to the Cmu burden, we administered clodronate liposomes 1 day before infection with pGFP-Luc-Cmu. Consistent with published evidence (20, 21), clodronate liposomes significantly depleted CD11b<sup>+</sup>Ly6G<sup>-</sup> blood and peritoneal monocytes/macrophages, and preserved CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils (**Figure 2C**). Macrophage depletion reduced Cmu burden, as the pGFP-Luc-Cmu signal was almost undetectable in the lower and upper genital tract 6 days pi (**Figure 2D**). These data indicate that macrophages and neutrophils take up Cmu in the inflamed lower genital tract and transport it systemically. After clodronate, dissemination to upper genital tract is minimal and the Cmu load is reduced.

### **Macrophages are necessary for Cmu-induced ReA**

To investigate the role of macrophages in the development of WT Cmu-induced ReA in SKG mice, macrophages were depleted with weekly injections of clodronate liposomes, starting on the day of Cmu genital infection. SKG mice infected with Cmu and treated with clodronate liposomes did not develop arthritis (**Figure 3A**), consistent with the reduction in genital tract Cmu burden (**Figure 2D**) and the relationship between Cmu burden and arthritis (2). Mean paw inflammatory indices were significantly decreased in infected SKG mice treated with clodronate liposomes when compared to SKG mice treated with PBS liposomes and no different from uninfected SKG mice. This was confirmed histologically: cellular infiltration was present in the skin, fore and hind paws of mice treated by PBS liposomes and not in mice treated with clodronate liposomes, with a reduced histological score at each site (**Figure 3B**). Enthesitis, synovitis and plantar fasciitis were also abrogated by macrophage depletion (**Figure 3C**). Anti-chlamydial MOMP antibody titers were not affected by clodronate

treatment (**Figure 3D**). This is consistent with evidence that CD4 T cells and neutrophils are necessary for anti-Cmu antibody production in BALB/c mice (22) and that anti-Cmu MOMP antibody titers are not reduced in SKG relative to BALB/c mice (2). These data indicate that Cmu transport by macrophages is required for the development of ReA but not Cmu antibody immune response in SKG mice.

### **Autophagy and ER stress characterize SKG response to Cmu infection**

Intracellular pathogens may enhance their replicative fitness in stressed host cells. To compare propensity of SKG and BALB/c mice to develop an IRG response and ER stress in response to Cmu infection, we quantified ER stress-related *Hspa5* and IRG-related *Tgtp1* (also known as *Irgb6*) expression in genital tract and ankle joint before infection and 1 and 5 weeks pi as arthritis began. In the joint, *Tgtp1* and *Hspa5* were constitutively increased in SKG mice. *Tgtp1* increased 100 fold in SKG mice 1 week pi (**Figure 4A**). *Hspa5* increased in BALB/c mice 5 weeks pi (**Figure 4B**). In the uterine horn, *Tgtp1* increased 10 fold in BALB/c mice 1 week pi then 100 fold 5 weeks pi in SKG mice (**Figure 4C**). *Hspa5* also increased in SKG mice at 5 weeks pi (**Figure 4D**). *Hspa5* and *Tgtp1* levels were strongly correlated in joint and uterine horn (**Figure 4E-F**). These data indicate that while BALB/c mice make a small early genital tract IRG response that drives autophagy to control chlamydia replication, the SKG response is much greater – first in joint and then in uterine horn. This IRG response correlates with an ER stress response in SKG genital tract after Cmu infection. In contrast and unrelated to infection, ER stress and low levels of IRG are constitutive in the joints of naïve SKG mice.

### **Persistent infection and inflammation are required for Cmu -induced ReA in SKG mice**

Dissemination of chlamydial PAMPs systemically promotes TLR2-mediated TNF production. The cryptic plasmid is a virulence factor for Cmu and *Chlamydia trachomatis* in animal models. The plasmid is required for TLR2 signaling and disease pathology (2). In BALB/c mice, a plasmid-cured strain of Cmu retained infectivity in the genital tract and generated an effective Th1 immune response. However, it failed to induce inflammatory disease of the oviduct or significant levels of genital tract TNF production. Furthermore bone marrow-derived DC infected with this strain *in vitro* secreted little TNF (23). Hence, we infected SKG mice with a plasmid-deficient Cmu

strain to ascertain the role of the plasmid in Chlamydia-mediated inflammation in ReA. While SKG mice infected with WT Cmu developed swelling of fore and hind paws, which gradually evolved into an asymmetrical polyarthritis, no joint swelling occurred after infection of SKG mice with plasmid-deficient Cmu over 12 weeks (**Figure 5A**). Rifampicin and doxycycline antibiotics that in combination have been shown to eliminate Cmu (2), begun 2 weeks after the onset of arthritis, significantly suppressed arthritis severity in SKG mice infected with Cmu (**Figure 5B**) indicating that long-term chlamydial infection drives ReA.

Given neutrophils and macrophages infiltrated the genital tract of Cmu infected mice and transported Cmu systemically, we sorted neutrophils (CD11b<sup>+</sup>Ly6G<sup>+</sup>), macrophages/DCs (MHCII<sup>+</sup>CD11c<sup>+</sup>) and lymphocytes (CD3<sup>+</sup>) from uterine horns and spleen at baseline and 1 week pi, and quantified the expression of *Il23a*, *Il17a* and *Il12b*. In the genital tract, expression of *Il23a* and *Il17a* was significantly higher in SKG neutrophils than either SKG macrophages/DCs or BALB/c neutrophils. In the spleen of SKG mice, neutrophils expressed higher *Il23a* and *Il17a* transcripts than macrophages/DC/T cells. *Il12b* was expressed by myeloid cells in genital tract, and at significantly greater levels by SKG macrophages/DC than SKG neutrophils or BALB/c macrophages/DC (**Figure 5C-E**). To test the function of SpA-associated cytokines in arthritis development, we blocked IL23p19 in WT Cmu-infected SKG mice, or infected *Il17a*<sup>-/-</sup> SKG mice. Early inhibition of IL-23p19 (from weeks 1 to 12) prevented ReA development whereas inhibition during the disease period from weeks 5 to 12 did not (**Figure 5F, G and Supplementary Figure 2A**). All mice treated with early inhibition of IL-23 had high titers of anti-MOMP antibodies (**Supplementary Figure 2B**). Early inhibition of IL-23p19 had no effect on vaginal shedding of Cmu (**Supplementary Figure 2C**). *Il17a*<sup>-/-</sup> SKG mice did not develop persistent arthritis after genital infection with Cmu (**Figure 5H**). Together these data suggest that plasmid-mediated Cmu PAMPs drive early expression of neutrophil and macrophage IL-23 and IL-17A in SKG genital tract and that depletion of either macrophages, IL-23, IL-17A or the plasmid is sufficient to block disease development.

### **Cmu ReA in SKG mice depends on TNF**

We showed previously in SKG mice depleted of Foxp3<sup>+</sup> regulatory T cells, that Cmu-induced ReA was TNF-dependent (2). In SKG mice, *Tnf* expression increased in the

joint at 1 week and persisted at 5 weeks pi, at the onset of arthritis (**Figure 6A**). This increase in *Tnf* in the joint coincided with increased expression in the genital tract 1-week pi in both mouse strains (**Figure 6B**). While *Tnf* transcripts were expressed by both macrophages and neutrophils in the uterine horn, SKG neutrophils expressed significantly greater levels than macrophages in the spleen at 1-week pi (**Figure 6C**). Inhibition of TNF with either monoclonal antibody or soluble decoy TNF receptor (scores combined) decreased joint inflammation in SKG mice infected with WT Cmu, whether the inhibition began at the time of infection or 5 weeks later, at disease onset (**Figure 6D and E**). TNF inhibition did not alter the levels of anti-MOMP antibodies in Cmu-infected SKG mice (**Figure 6F**) or vaginal shedding of Cmu (**Supplementary Figure 2D**). These data suggest that infected macrophages and neutrophils drive arthritis onset by TNF production.

## Discussion

We show here that the load of Cmu is higher in SKG than BALB/c mice. While proinflammatory neutrophils appear to contribute to disease initiation, macrophage depletion reduces Cmu tissue dissemination, tissue burden, and arthritis, but not anti-MOMP antibodies. Our data suggest that enhanced bacterial survival in SKG macrophages drives production of TNF required for persistent arthritis. Macrophages are known to transport Cmu to other tissues (24). In SKG mice, Cmu DNA is transported predominantly by macrophages, as well as neutrophils, to spleen after genital tract infection. In SKG mice, depletion of macrophages by clodronate prevents Cmu dissemination and disease. Although arthritis fails to develop with macrophage depletion in Cmu infected SKG mice, anti-MOMP antibodies, which require neutrophils and B cells, are still produced (22). In contrast, BALB/c mice clear Cmu even when macrophages are present, and fail to develop arthritis or a TNF response in the joint. Consistent with this, the load of Cmu is higher in SKG than in BALB/c mice, and controlling the growth of chlamydia with a combination of antibiotics controls the disease. Although Clodronate liposomes have been shown to selectively deplete macrophages and not conventional DC or neutrophils (25), the dependence of ReA on macrophages would ideally be confirmed in *Csf1r*-DTR mice on the SKG genetic background. Together, our data indicate that poor chlamydia clearance by macrophages underpins ReA in SKG mice. The proinflammatory impact of macrophages and poor clearance of Cmu in SKG mice is consistent with the

production of TNF by macrophages and impaired production of MOMP-specific T cell IFN- $\gamma$  and IL-17 (2), which are required for Cmu control (26, 27).

ER stress and proinflammatory cytokines also play an important role in Cmu-induced ReA in SKG mice. ER stress was constitutively increased in SKG joints and induced by Cmu infection of the genital tract, along with a delayed and excessive IRG response. We focused on *Tgtp1* as a marker of autophagy because it is implicated in the initiation and coordination of the routing of other IRGs into the early chlamydial inclusions independent of IFN- $\gamma$  stimulation, and plays a necessary role in the control of Chlamydial replication (15). In SKG mice, MOMP-specific T cells produce low levels of IFN- $\gamma$  (2). Expression of *Il23a* and *Il17a* was increased in SKG uterine and splenic neutrophils at the time that *Tgtp1* increased in the joint. These data suggest a relationship between infection-driven autophagy to control Cmu transported to the joint by macrophages and infection-associated IL-23/IL-17 in the genital tract. IL-23 and IL-17 drive arthritis development, as anti-IL-23p19 administered during infection or *Il17a* deficiency suppressed arthritis. Similarly, matrix metalloproteinase production by neutrophils is decreased in Cmu-infected *Il17<sup>-/-</sup>* mice, along with reduced oviduct pathology (28). The inter-relationship between infection, ER stress, autophagy and IL-23 in SpA is supported by: induction of the unfolded protein response (UPR), autophagy and thus host cell survival upon Cmu infection, the relationship between autophagy and IL-23 in AS gut, the sequestration of A20 by autophagy and the provocation of IL-23 production by ER stress in the context of TLR stimulation (6, 12, 13, 29). Thus arthritis pathogenesis after Cmu infection of SKG mice appears to be two-pronged, with neutrophils contributing to disease initiation and macrophages bearing the pathogen burden for persistent pathogenic TNF production. It is interesting that *Tgtp1* and *Hspa5* increased at 5 weeks pi in the uterine horn of SKG mice, suggesting that *Chlamydia*-infected macrophages may continue to be a source of reinfection and thus perpetuation of inflammation.

Here we show that TNF inhibitors, both monoclonal antibody and soluble receptor, suppress inflammatory arthritis in SKG mice, whether TNF is blocked at the time of Cmu infection or at arthritis onset 5 weeks later, similar to human ReA (30). Furthermore arthritis development depends on the Cmu cryptic virulence plasmid that is required for TLR2-mediated TNF production upon dissemination (2). Together our

data support the concept that Cmu ReA results from host factors promoting ER stress, bacterial virulence factors promoting inflammation under stress and host immune dysregulation underlying poor pathogen control.

Previous studies identified DNA and other bacterial products from multiple species implicated in ReA, including *Salmonella typhimurium* and *Chlamydia trachomatis*, in the synovial tissues and fluid of ReA patients, and found the persistent forms to be metabolically-active (31). Dissemination of Cmu to sites outside the genital tract, such as spleen, implies the dissemination of PAMPs and capacity for presentation of chlamydial antigen to T cells, driving a chronic inflammatory response. Indeed, *Chlamydia*-specific CD4+ and CD8+ T cells have been identified in the joints of patients with *Chlamydia*-induced ReA (31). Persistent *Chlamydia* and chlamydial antigen and TLR signals will drive macrophage and DC activation, chronic activation of T cells and production of pro-inflammatory cytokines, thus inducing persistent inflammation (32, 33).

Persistent *Chlamydia trachomatis* and *Chlamydia pneumoniae* are in the aberrant-body phase, with an aberrant morphology and metabolic state (34, 35). Bacteria in this persistent state are non-culturable by standard methods, resistant to antibiotics and display an unusual transcription profile (36, 37). *Chlamydia trachomatis* may enter a persistent state after infection of monocytes, influenced by the macrophage polarization state (32). Persistent forms of *Salmonella typhimurium* are similarly induced by macrophage internalization (38), and are related to genomic toxin-antitoxin systems, which increase bacterial fitness under stress conditions after macrophage internalization, antibiotic exposure and/or viral co-infection. While toxin-antitoxin systems remain quiescent under favorable growth conditions, toxins can be activated in response to stress, suppressing bacterial growth and promoting a stress-tolerant dormant state. The increased ER stress in joints and infection-induced ER stress in the genital tract of SKG mice would thus favour the persistence of Cmu or *Salmonella typhimurium*.

Autophagy plays a major role in the processing of *Chlamydia trachomatis* in macrophages (16, 39), to restrict the formation of infectious inclusion bodies, with potential impact on IL-23 production. Autophagy restricts *Chlamydia trachomatis*



growth in human macrophages via IFN-inducible IRG proteins (15, 16). IFN- $\gamma$  is fundamental to the eradication of intracellular pathogens through macrophage activation, nitric oxide, reactive oxygen species and indoleamine 2,3-dioxygenase production and tryptophan starvation (16, 40, 41). The impaired production of IFN- $\gamma$  by SKG mice T cells in response to chlamydial antigen and impaired macrophage responsiveness to IFN- $\gamma$  in SpA are thus significant (2, 42, 43). Moreover, autophagy mediates bacterial killing in macrophages (44) and contributes to antigen presentation and IL-23 secretion (12, 45, 46). Various cytokines have been shown to stimulate autophagy in macrophages including TNF, IFN- $\gamma$ , IL-1 $\alpha$  and IL-1 $\beta$  (44). While autophagy activation in human AS may be the consequence of HLA B27 misfolding in the gut (12), in SKG mice, activation of autophagy may be the consequence of macrophage infection by Cmu, and may help to drive IL-23.

IL-23 not only supports the differentiation of Th17 cells but is also required for the secretion of IL-17, IFN- $\gamma$  and IL-22 by Th17 and  $\gamma\delta$  T cells (47, 48). Since IL-23 blockade prevented arthritis only during infection, IL-23 must drive disease pathogenesis prior to development of arthritis, while TNF mediates clinical inflammatory manifestations. This is supported by evidence that IL-23 is only involved early in the pathogenesis of autoimmune inflammatory animal models, promoting the development of inflammatory pathogenic antibodies (49) and that blocking this cytokine during established AS is ineffective (50). Collectively, our results identify the pivotal role played by macrophages in the development of Cmu-induced ReA in SKG mice.

### Figure legends

#### **Figure 1. Macrophages and neutrophils infiltrate the genital tract of infected mice and transport *Chlamydia muridarum* systemically**

Female BALB/c or SKG mice (n=2-4/group) remained untreated or were primed with progesterone, then infected 1 week later with  $10^6$  IFU *Chlamydia muridarum* (Cmu). One week post-infection, flow cytometric analysis of number (**A, B, C**) of live CD45.2<sup>+</sup> dendritic cells (MHCII<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>CD103<sup>+</sup>), macrophages (MHCII<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>CX3CR1<sup>+</sup>), and neutrophils (MHCII<sup>-</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup>) were assessed. Cmu DNA detected by PCR (**D**) in splenic dendritic cells (DC)

(MHCII<sup>+</sup>CD11c<sup>+</sup>CD103<sup>+</sup>), macrophages (Mac) (MHCII<sup>+</sup>CD11c<sup>+</sup>CX3CR1<sup>+</sup>), and neutrophils (Neu) (MHCII<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup>). To quantify Cmu DNA, Cmu copy number was normalized to number of sorted cells (**E**). Data represent mean  $\pm$ SEM. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001, with 2-way ANOVA and post hoc Tukey's test.

### **Figure 2. Infected macrophages disseminate *Chlamydia muridarum* to upper genital tract**

SKG and BALB/c mice were infected intra-vaginally with  $2 \cdot 10^5$  IFU pGreen Fluorescent Protein-Luciferase-*Chlamydia muridarum* (Luc Cmu) weiss strain. Infected and control uninfected mice were imaged *in vivo* 7 and 11 days post infection (pi) (**A**). Cmu load in genital swabs from infected mice from day 3 to day 15 pi with  $10^6$  IFU wild type (wt Cmu) (left) or Luc Cmu (right,  $n=5$  per group) (**B**). SKG and BALB/c mice were administered PBS or clodronate liposomes the day before infection with  $10^6$  Luc Cmu. Proportion of macrophages/monocytes (CD11b<sup>+</sup>Ly6G<sup>-</sup>) and neutrophils (CD11b<sup>+</sup>Ly6G<sup>+</sup>) were assessed in blood and peritoneal cavity 3 days after clodronate injection by flow cytometry ( $n=7$  mice) or PBS liposome ( $n=6$  mice) (**C**). Mice were imaged *in vivo*, 6 days later (**D**). Representative of 2 similar experiments. Data represent mean  $\pm$ SEM. \*\* $p$ <0.01, \*\*\*\* $p$ <0.0001, (**B**) 2-way ANOVA and post hoc Tukey's test; (**C**) Mann-Whitney test.

### **Figure 3. Macrophages are immunosuppressive and required for *Chlamydia muridarum* reactive arthritis**

SKG mice were administered PBS ( $n=7$ ) or clodronate liposomes ( $n=7$ , 2mg/20g mouse as initial dose then 1mg/20g/week) from the day of *Chlamydia muridarum* (Cmu) infection until week 12. Inflammation index indicates mean  $\pm$  SEM ankle width (mm) variation compared to baseline (**A**). Hind paw joint and skin histological scores (0-5) 12 weeks post infection (**B**). Representative H&E-stained sections from heels, hind paw joints and ear skin, harvested 12 weeks after infection (**C**). Original magnification x4 for heels, hind paw, and x20 for ear skin. Bars represent 500 $\mu$ m for magnification x4 and 100 $\mu$ m for x20. Arrows represent Achilles tendinitis, synovitis, fasciitis, inflammatory cell infiltrate and epidermal thickening. Serum anti-Cmu MOMP antibody titers, measured by ELISA, 12 weeks post-infection (**D**). \* $p$ <0.05, \*\*\* $p$ <0.001, with 2-way ANOVA and post hoc Tukey's test (**A**), t-test (**B**) and one-way non-parametric ANOVA (Kruskal Wallis test) and post hoc Dunn's test (**D**).

**Figure 4. Endoplasmic reticulum stress and immunity-related GTPase are increased in SKG joints and genital tract**

*Tgtp1* (A, C) and *Hspa5* (B, D) were quantified by RT-qPCR analysis relative to non infected BALB/c mice at baseline and 1 and 5 weeks post-infection (pi) in joints (A, B) and uterine horn (C, D) of infected SKG (black circle) and BALB/c (white circle) mice. Correlation between Ct *Tgtp1* and Ct *Hspa5* in joint (E) and uterine horn (F) of infected SKG mice. Each symbol represents an individual mouse (n=3 to 7 mice per group). Data represent mean  $\pm$  SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, with one-way ANOVA and post hoc Tukey's test.

**Figure 5. Neutrophil derived IL-23 and IL-17 promote *Chlamydia muridarum* reactive arthritis**

Inflammation index over 12 weeks of SKG mice infected with Cmu or Plasmid-deficient Cmu (A). Inflammation index over 12 weeks of SKG mice infected with Cmu and treated with rifampicin (0.4 mg/day) and doxycycline (0.3 mg/day) from week 7 to week 9 post infection (pi) (B). BALB/c or SKG mice were infected with Cmu. One-week pi, macrophages/DCs (MHCII<sup>+</sup>CD11c<sup>+</sup>), neutrophils (CD11b<sup>+</sup>Ly6G<sup>+</sup>) and lymphocytes (CD3<sup>+</sup>) were sorted from spleen and uterine horn. Expression of *Il23a* (C) *Il17a* (D) and *Il12b* (E) relative to *Hprt* was quantified by RT-qPCR (n=3). Inflammation index over 12 weeks of Cmu-infected SKG mice administered anti-mouse IL23p19 or isotype mAb either from week 1 to week 12 (w1-w12, n=9) or from week 5 to week 12 (w5-w12, n=8, isotype n=13) (F). Hind paw histological scores from Cmu-infected mice treated with anti-mouse IL23p19 or isotype mAb (G). Inflammation index over 12 weeks of Cmu-infected SKG and *Il17a*<sup>-/-</sup> SKG mice. (n=4 to 6 per group) (H). Data represent mean  $\pm$  SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, (A, B, F, H) 2-way ANOVA; (C, D, E, G) one-way ANOVA and post hoc Tukey's test.

**Figure 6. *Chlamydia muridarum* reactive arthritis depends on TNF**

*Tnf* expression was quantified by RT-qPCR analysis relative to non infected BALB/c mice at baseline, 1 and 5 weeks post infection (pi) in joints (A) and uterine horn (B) of infected SKG (black circle) and BALB/c (white circle) mice. BALB/c or SKG mice were infected with *Chlamydia muridarum* (Cmu). One-week pi, macrophages/DCs (MHCII<sup>+</sup>CD11c<sup>+</sup>), neutrophils (CD11b<sup>+</sup>Ly6G<sup>+</sup>) and lymphocytes (CD3<sup>+</sup>) were sorted

from spleen and uterine horn. Expression of *Tnf* relative to relative to *Hprt* was quantified by RT-qPCR (**C**). Inflammation index over 12 weeks of Cmu-infected SKG mice administered with mouse-adapted certolizumab pegol or etanercept or PBS either from week 1 to week 12 (w1-w12, n=18, treatments pooled) or from week 5 to week 12 (w5-w12, n=17, treatment pooled) (PBS n=19) (**D**). Hind paw histological score in mice 12 weeks pi with Cmu and treated with anti-TNF or PBS (**E**). Serum anti-Cmu MOMP antibody titres, measured by ELISA, 12 weeks pi (**F**). Bars represent the mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ , (**A, B, C, E**) one-way ANOVA and post hoc Tukey's test; (**D**) 2-way ANOVA and post Tukey's test; (**F**) one-way non-parametric ANOVA (Kruskal Wallis test) and post hoc Dunn's test.

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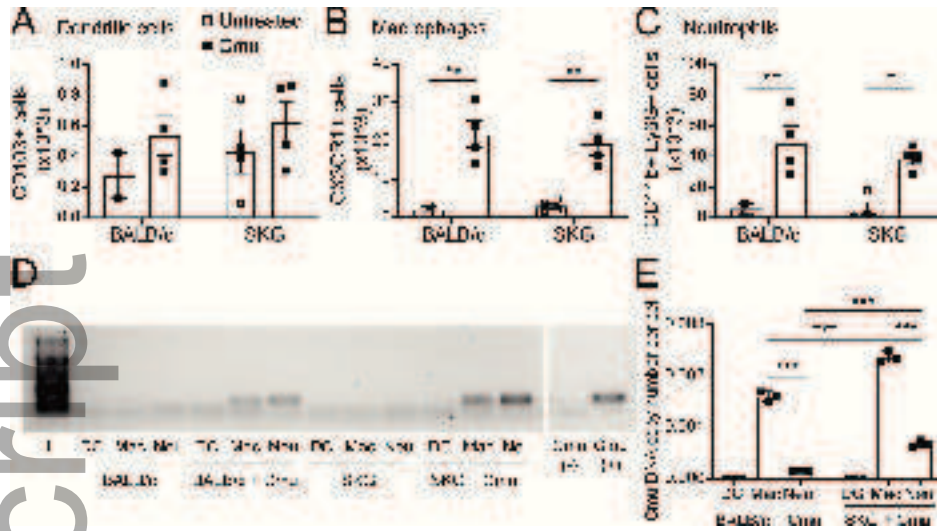
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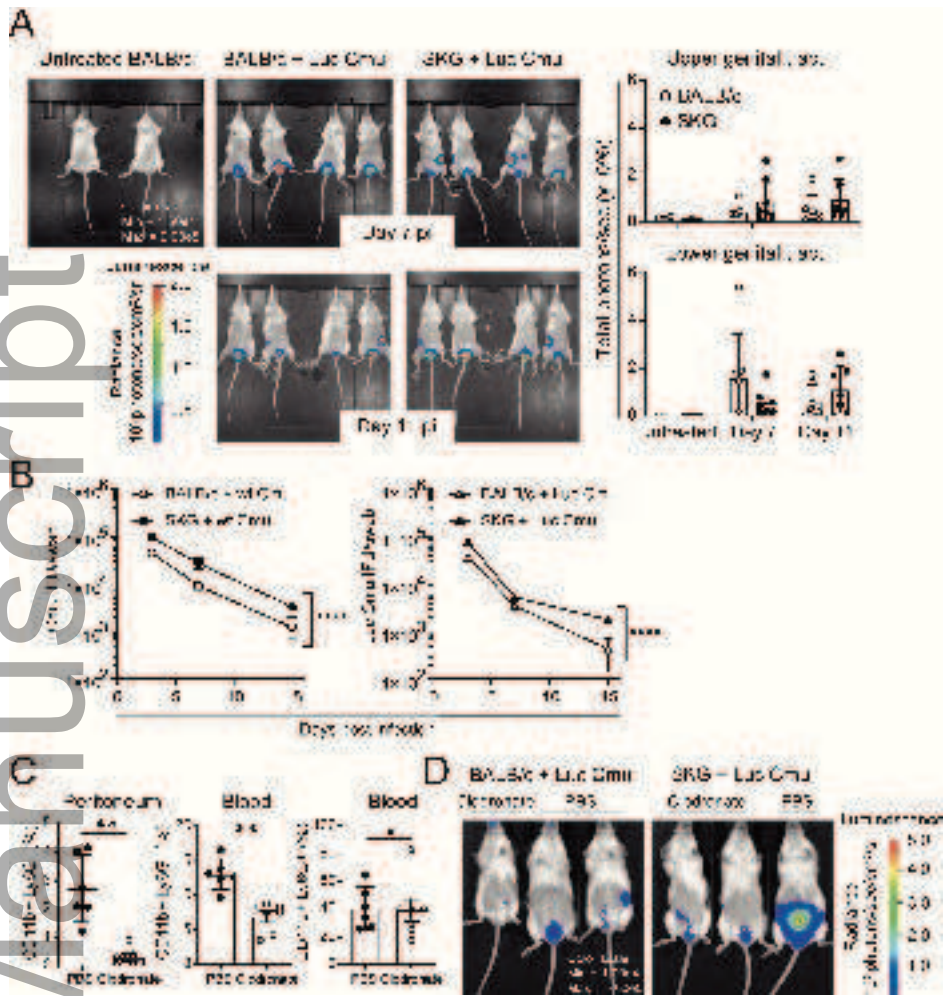


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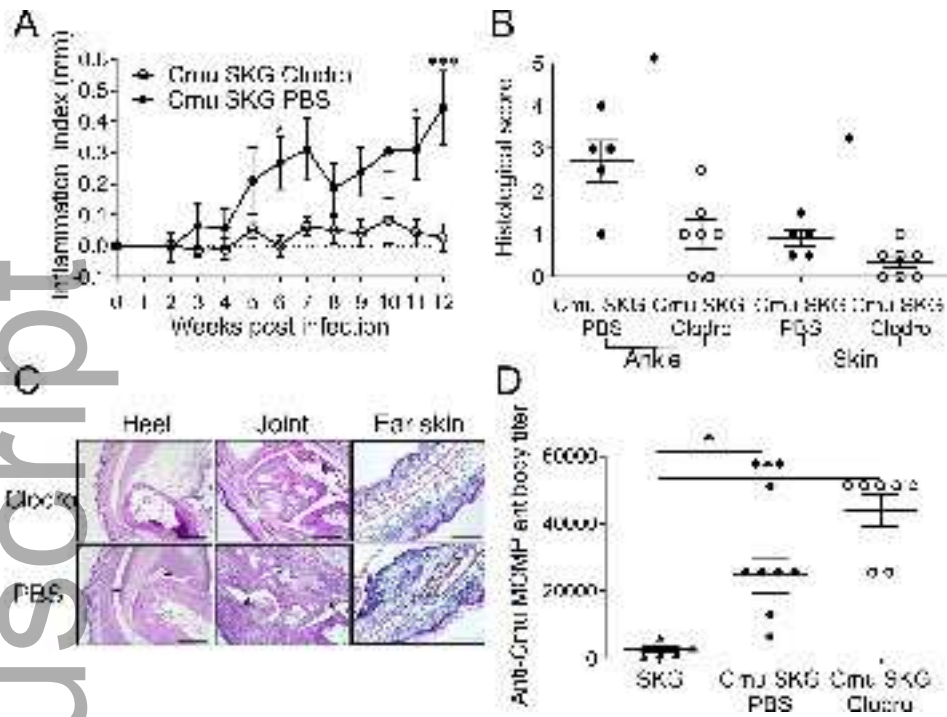
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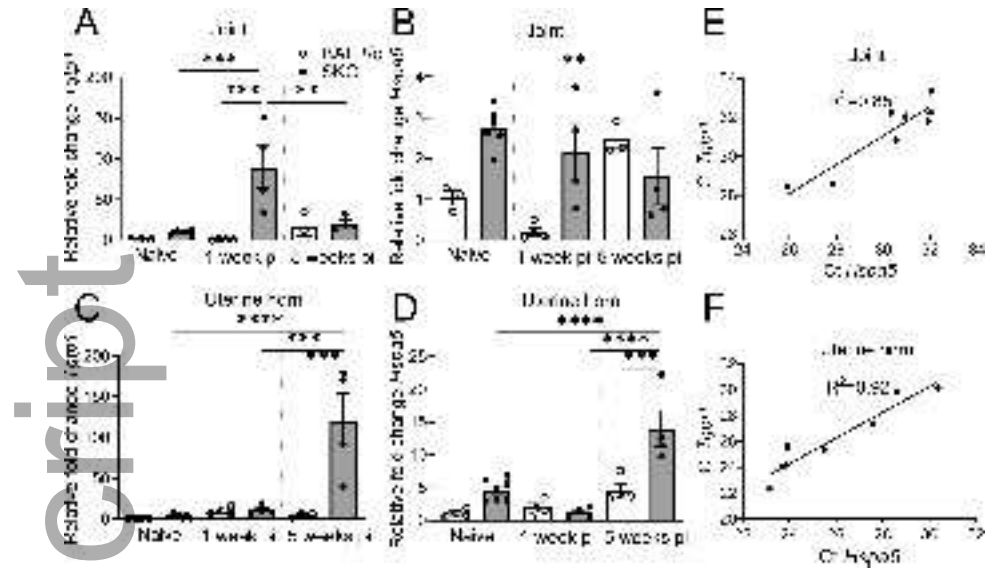
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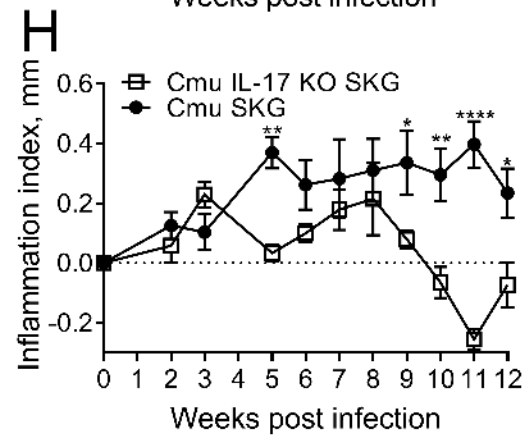
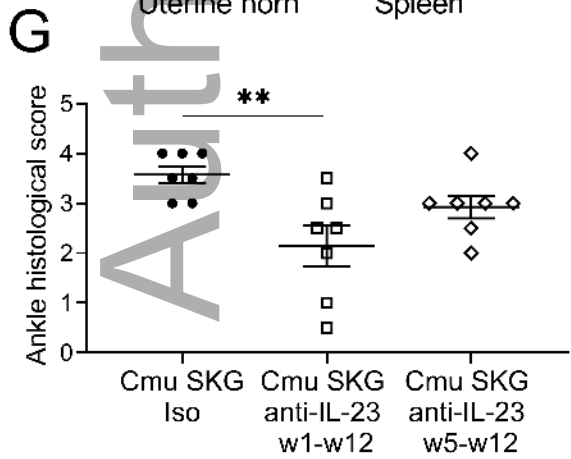
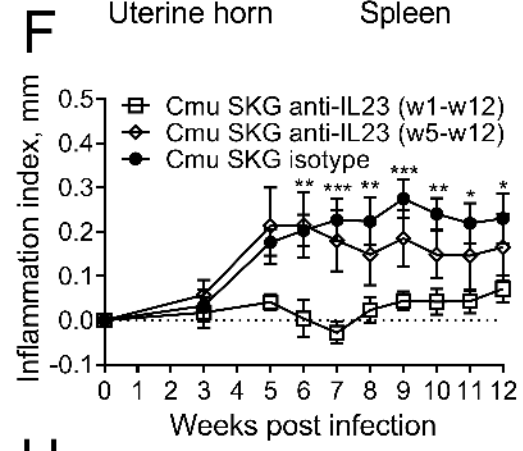
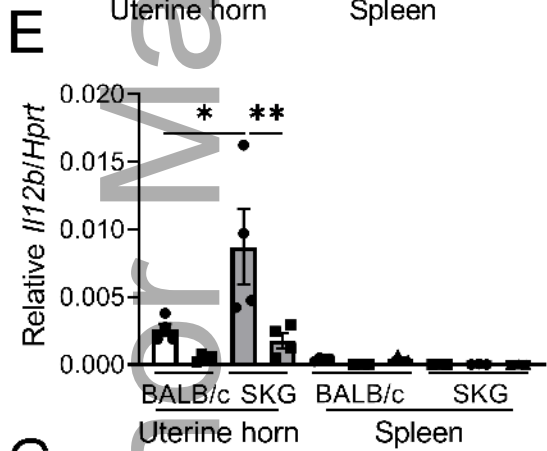
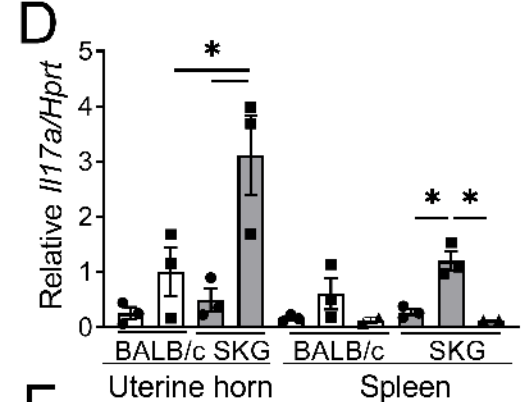
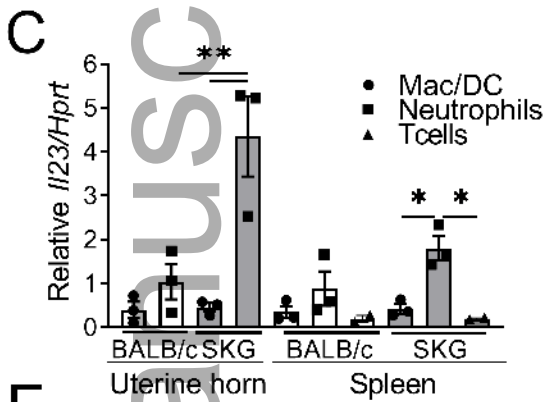
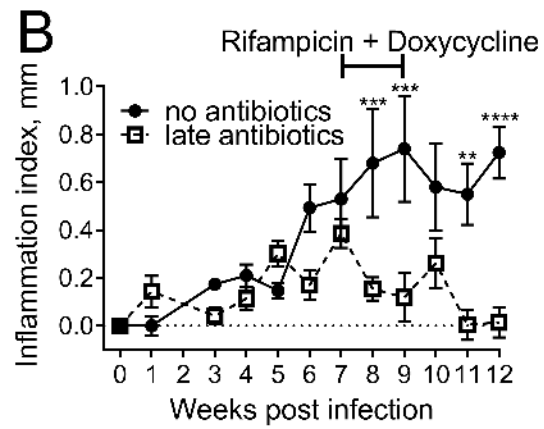
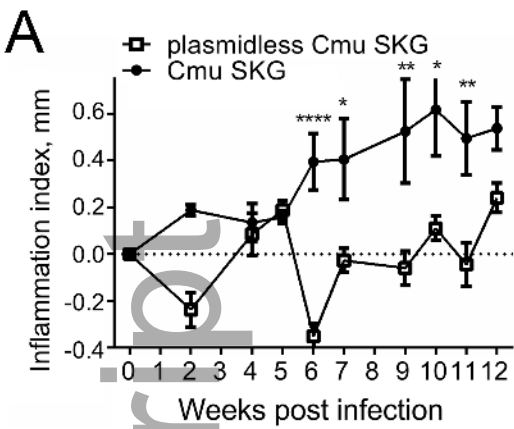
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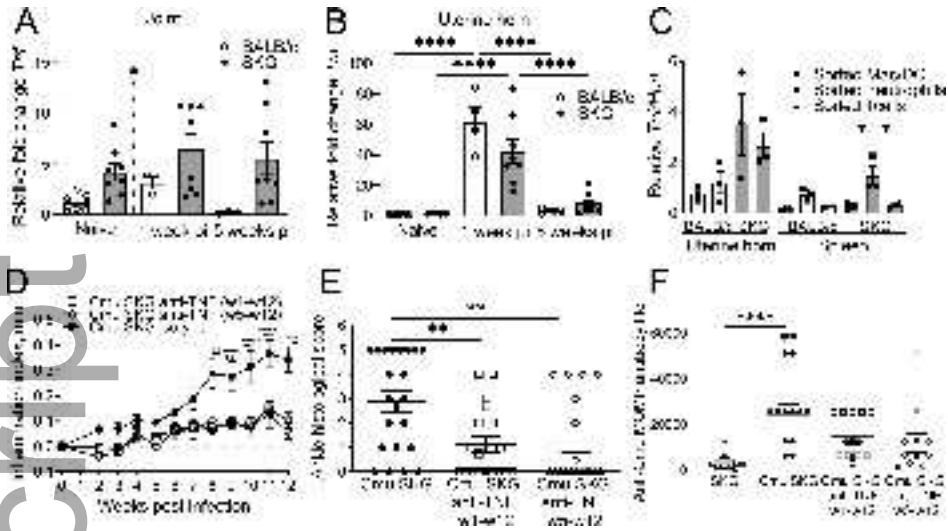


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art\_41653\_f4.tif





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