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Evaluation of interleukin-17D in host immunity to group A *Streptococcus* infection

Allen Washington Jr.[*], **Nissi Varki**[*,†], **J. Andrés Valderrama**[‡], **Victor Nizet**[‡,§], **Jack D. Bui**[*]

[*]Department of Pathology, University of California at San Diego, 9500 Gilman Dr. MC 0612, La Jolla, CA 92093, USA

[†]Glycobiology Research and Training Center, University of California at San Diego, 9500 Gilman Dr. MC 0612, La Jolla, CA 92093, USA

[‡]Department of Pediatrics, University of California at San Diego, 9500 Gilman Dr. MC 0612, La Jolla, CA 92093, USA

[§]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, 9500 Gilman Dr. MC 0612, La Jolla, CA 92093, USA

Abstract

Interleukin-17D (IL-17D) is a cytokine that belongs to the IL-17 family and is conserved in vertebrates and invertebrates. In contrast to IL-17A and IL-17F, which are expressed in Th-17 cells, IL-17D is expressed broadly in non-immune cells. IL-17D can promote immune responses to cancer and viruses in part by inducing chemokines and recruiting innate immune cells such as natural killer (NK) cells. Although bacterial infection can induce IL-17D in fish and invertebrates, the role of mammalian IL-17D in anti-bacterial immunity has not been established. To determine whether IL-17D has a role in mediating host defense against bacterial infections, we studied intraperitoneal infection by group A *Streptococcus* (GAS) in wild-type (WT) and *Il17d*$^{-/-}$ mice. Compared to WT animals, mice deficient in IL-17D experienced decreased survival, had greater weight loss, and showed increased bacterial burden in the kidney and peritoneal cavity following GAS challenge. In WT animals, IL-17D transcript was induced by GAS infection and correlated to increased levels of chemokine CCL2 and greater neutrophil recruitment. Of note, GAS-mediated IL-17D induction in non-immune cells required live bacteria, suggesting that processes beyond recognition of pathogen-associated molecular patterns were required for IL-17D induction. Based on our results, we propose a model in which non-immune cells can discriminate between non-viable and viable GAS cells, responding only to the latter by inducing IL-17D.

Keywords

Group A Streptococcus; IL-17D; Ccl2; neutrophils

Address correspondence to: Jack D. Bui, Department of Pathology, University of California, 9500 Gilman Dr MC 0612, La Jolla, CA 92093-0612; Phone 858-534-3890; Fax 858-822-4566; jbui@ucsd.edu.

Introduction

Interleukin (IL)-17 cytokines include six members (A-F) that exhibit functional roles in inflammation, autoimmunity, and cancer (1, 2). In contrast to IL-17A and IL-17F that are mostly expressed in T cells, IL-17D is expressed broadly outside of the hematopoietic compartment and has limited expression in bone marrow, thymus, and spleen (3). IL-17B and IL-17C are also expressed in non-immune cells and induce similar sets of downstream genes as IL-17A (3–6). IL-17A and IL-17C protect the host from various bacterial species in experimental infection models (7–11). IL-17E (aka IL-25), which is unlike other cytokines in the family, promotes T helper type 2 immune responses and mediates immunity against parasites (12). IL-17B, the least understood IL-17 member with respect to pathogen responses, antagonizes IL-17E signaling and thus may counteract excessive inflammatory processes (13).

We previously found IL-17D to have roles in anti-tumor and anti-viral immunity via induction of the chemokine CCL2 and recruitment of NK cells (14–18). Additionally, we and others have shown that IL-17D induces some overlapping sets of genes downstream of IL-17A including IL-8 and CCL2 (3, 14). In experimental tumor models, exogenous or tumor-expressed IL-17D was sufficient to cause growth delay or tumor rejection (14, 15). IL-17D is regulated by the oxidative stress sensor nuclear factor erythroid-derived 2-like 2 (Nrf2) (16), and we have previously described that oxidative stressors and viral infection activate Nrf2, triggering IL-17D expression and immune cell recruitment (16–18). *Il17d*$^{-/-}$ mice are consequently more susceptible to murine cytomegalovirus (MCMV) infection and carcinogen-induced tumors (16, 17).

The role of IL-17D in anti-bacterial immunity is relatively unexplored in mammals, but studies of IL-17D homologs in fish and invertebrate animal models have suggested a protective role (19–23). In fish, *Il17d* is expressed constitutively and further increased in response to gram negative bacterial challenge in organs such as the skin and head kidney (19, 20); similar responses have been demonstrated in marine invertebrates, such as oysters and amphioxus (23, 22). In addition, both gram negative live bacteria and lipopolysaccharide (LPS) induce *Il17d* in skin cells of lampreys *in vitro* (21).

In mammals, IL-17 cytokines such as IL-17A and IL-17F are induced in CD4^{+} T cells by retinoic acid-related orphan nuclear receptor, RORγt and RORα (24–26), epithelial cell IL-17C is induced in response to Gram-negative bacteria (4, 27), Similarly, IL-17E in epithelial cells is induced upon detecting extracellular helminths through succinate (28, 29). In mice, we found IL-17D to be induced by viral infection, constitutively expressed in immunogenic cancer cells, and directly induced by the anti-oxidant transcription factor Nrf2 (16, 17, 14). It is not clear whether bacterial infection or components induce IL-17D in mammalian systems.

Streptococcus pyogenes, also known as group A *Streptococcus* (GAS), is a leading Gram-positive human bacterial pathogen associated with a wide range of pathological outcomes, from simple pharyngitis and skin infection, to invasive necrotizing fasciitis and toxic shock syndrome, and importantly, to post-infectious sequelae including rheumatic heart disease

and glomerulonephritis (30–32). GAS expresses pore-forming toxins (including streptolysins S and O) and extracellular proteases that can induce cellular damage and organ system dysfunction during invasive infection (33, 30). A robust inflammatory response to GAS infection is catalyzed by release of cytokines by immune cells that sense bacterial components or cellular damage molecules (34, 35). GAS infection also stimulates generation of reactive oxygen species (ROS) (36, 37), a cellular mechanism of inhibition for bacterial replication that simultaneously promotes inflammatory cell death (38, 39, 34). Since ROS are known inducer molecules of IL-17D, we hypothesized that IL-17D would be induced and potentially play a protective role during GAS infection.

Materials & Methods

Mice

The UCSD Institutional Animal Care and Use Committee approved all animal use and procedures (protocol #S06201). C57BL/6 and IL-17D-deficient (16, 17) mice were housed in specific pathogen-free conditions prior to use. Mice were between 6 and 12 weeks old and were age- and sex-matched across experiments.

In vivo group A Streptococcus (GAS) murine infection model

WT GAS strain 5448 was isolated from a patient with necrotizing fasciitis and toxic shock syndrome (40) and is representative of the globally disseminated serotype M1T1 clone that is the predominant cause of life-threatening invasive GAS infections (41). Mice were injected intraperitoneally with the indicated inoculum of GAS in a total volume of 200μL phosphate buffered saline (PBS). Each inoculum was prepared by growing GAS overnight in Todd-Hewitt broth (THB, Teknova) and adjusting the concentration according to the spectrophotometric O.D. 600 reading (35). Inoculum colony forming units (CFU) were confirmed by serial dilution plating on THB agar (THA). One-time injection of 200mg/kg N-Acetyl-L-cysteine (NAC) in PBS was conducted 4 h prior to GAS infection. Anti-CCL2 polyclonal antibody or control IgG (R&D) was co-injected with GAS. For survival assessment, mice were monitored at least every 12 h to record the body weight and survey health status including signs of lethargy.

Peritoneal exudate and cells were collected by lavage with 2.5mL PBS. Biopsies of kidney, spleen, lung, and the heart of each mouse was collected to determine GAS burden. The tissues were weighed and homogenized in 1mL PBS or RPMI medium containing 2% FBS through a 70μm strainer. Homogenized tissues were serially diluted 10-fold, and 50μL or 10μL from each aliquot were cultured overnight on THA at 36.5°C. The measurement of CFU/g was determined based on total CFU in 1mL divided by the weight of organ; samples without a colony were arbitrarily assigned as 1 CFU/mL and divided by the weight of the organ in order to provide graphable non-zero data points.

In vitro group A Streptococcus (GAS) infection of cultured cells

Heat-killed GAS were prepared by placing bacteria at 65–75°C for 5–10 min; absence of CFU growth was confirmed by plating aliquots on THA. The multiplicity of infection (MOI) of GAS-to-cultured cells (immune and non-immune cells) was 1:1 unless specified.

Peritoneal exudate cells (PEC, ~30% macrophages and ~40% B cells), F244 fibrosarcoma, and HEK293 cells were cultured in RPMI containing 10% FBS with penicillin and streptomycin and switched to 2% FBS without antibiotics the day before the experiment. PECs were collected by lavage using 2.5mL PBS or FACS buffer per peritoneum. Cell culture plates were spun at 1500 rpm for 5 min to facilitate contact between GAS and cultured cells. Cells were pre-treated for 1 h with either N-Acetyl-L-cysteine (NAC) or ammonium pyrrolidine dithiocarbamate (PDTC) at a concentration of 0.6 μM and 0.4 μM respectively (Sigma). Final concentration of peptidoglycan was prepared as 1μg/mL (Invivogen).

Flow cytometry experiments

Percentages of leukocytes within each sample were determined using a BD FACSCANTO flow cytometer. CD45 (30-F11), Ly6G (1A8) markers were employed to identify neutrophils; CD45, CD11b^{mid} (M1–70) for monocytes; CD45, CD11b^{high}, F4/80 (BM8) for macrophages; NK1.1 (PK136) for NK cells; CD3 (145–2C11) for T cells; CD45R (RA3–6B2) for B cells (all from Biolegend). Anti-CD192 (SA203G11) was used to identify CCR2-positive cells (Biolegend). 7AAD-positive cells were used to identify dead cells (Calbiochem).

Quantitative real-time PCR

RNA was extracted using TRI-zol (Life Technologies) or by using the Direct-zol RNA MiniPrep Kit (Zymogen). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems); qPCR data were generated using SYBR Green mix (Applied Biosystems) with forward and reverse primers of indicated genes; reaction was run on the One Step Plus Real-Time PCR System (Applied Biosystems). Ct values of *Hprt* or *Gapdh* reference gene of the vehicle or non-treated control samples were used in calculating the ΔΔCt in order to determine the $2^{-\Delta\Delta Ct}$ values. Primer sequences are listed in Supplemental Table 1.

Enzyme-linked immunosorbent assay

Biopsies of kidney from WT and Il17d$^{-/-}$ mice were weighed and homogenized in 1mL PBS through a 70μm strainer. Tissue lysates were prepared by using RIPA buffer (Teknova) with protease inhibitor and samples within the same experiment was normalized using Pierce BCA Protein Assay kit (Thermo Fisher). Protein CCL2 was measured using a Quantikine ELISA kit (R&D) following the manufacturer's protocol. Standard curve was generated and applied to calculate pg/mL CCL2 and fold difference was determined over mock infection.

Cytokine injection

C57BL/6 mice were maintained under specific pathogen-free conditions. 0.5μg of IL-17D or IL-17A (R&D) in 250μL PBS or vehicle in PBS were i.p. injected in each mouse. Bioactivity of these cytokines was confirmed previously in vivo (14). Heat-inactivated IL-17D was prepared by heating at 60°C for 30 min. Mice were euthanized using carbon dioxide, and peritoneal cavity was lavaged several times in total 10mL PBS containing 1%

FBS. Cells were incubated in RBC lysis buffer (Biolegend) and counted using the hemocytometer. In order to quantitate chemokine transcripts in mesothelial cells, peritoneal wall tissue was minced with scissors prior to the extraction of RNA. In order to analyze the mesothelial cell composition by flow cytometry, peritoneal wall tissues were minced with scissors and digested with collagenase (Worthington) in medium overnight at 36.5°C.

Statistical analysis

All statistical analyses were processed through a statistical software package (GraphPad Prism). Kaplan-Meier survival curves were generated and analyzed using the log-rank test in order to assess statistical significance. CFU data from organ homogenates and lavage were analyzed using the nonparametric Mann-Whitney *U* test. In vitro GAS incubation experiments were analyzed using the Student's two-tailed t-test. All P-values less than 0.05 were considered to be significant. ns = not significant. P values are denoted in figures as: *, $P<0.05$; **,$P<0.01$; ***,$P<0.001$.

Results

Recombinant IL-17D induces ccl2 and recruitment of innate immune cells into the peritoneum

We hypothesized that IL-17D promotes rapid immune responses in part by inducing chemokines such as CCL2 and recruiting neutrophils. To establish our *in vivo* model system, we first tested the response of C57BL/6 wild type mice to administration of purified recombinant IL-17D into the peritoneal space. Injection of IL-17D induced significant amounts of *Ccl2* in peritoneal wall tissue (i.e., mesothelial cells) within 2 hours (Fig. 1A). Other chemokines such as *Cxcl3* and *Ccl7* were also induced significantly after i.p. IL-17D injection (Fig. S1A). Injection of recombinant IL-17D also led to recruitment of early innate responders such as neutrophils and monocytes, which express CCR2 (Fig. 1B, Fig. S1B, Fig. S1C). Accumulation of neutrophils peaked at 4 hours, was induced also by IL-17A, and did not occur with heat inactivated IL-17D (Fig. 1B, Fig. 1C, Fig. S1D). Some chemokines that are induced by IL-17D, such as *Ccl2* and *Ccl7*, can also be induced by i.p. IL-17A injection and led to similar recruitment of cells such as monocytes (Fig. S1E, Fig. S1F), confirming that the peritoneal space could respond to IL-17 family cytokines. Notably, the peritoneal wall cells responding to IL-17 family cytokines are likely resident mesothelial cells, since we did not detect significant levels of immune cells within the peritoneal wall by flow cytometry (Fig. S1G).

Il17d$^{-/-}$ mice display slightly compromised responses to bacterial infection

Having established that non-immune cells in the peritoneum could respond to IL-17D, we next assessed the requirement for IL-17D in anti-bacterial responses where the bacteria are introduced into the peritoneum (i.p.). After i.p. infection with group A *Streptococcus* (GAS), we monitored WT and Il17d$^{-/-}$ mice for survival and weight loss. We observed slightly higher weight loss in Il17d$^{-/-}$ mice infected by GAS (Fig. 2A, Fig. 2B). We also observed a lower survival rate of Il17d$^{-/-}$ mice at the maximum GAS dose tested (5×10^7), but this did not achieve statistical significance (Fig. 2C).

Previously we had observed that Il17d$^{-/-}$ mice manifest higher viral burden in some organs after murine cytomegalovirus (MCMV) infection (17). Therefore, we examined bacterial burden in multiple tissues after GAS infection. Indeed, the kidney and peritoneal cavity harbored significantly higher GAS colony forming units (CFU) in Il17d$^{-/-}$ mice (Fig. 3A, Fig. 3B, Fig. S2A). The higher bacterial burdens in the kidneys of Il17d$^{-/-}$ animals was confirmed through three different challenge doses of GAS (Fig. 3C). The difference in bacterial burden was largest at 24 h and not at later times (Fig. S2B). We did not detect significant differences in bacterial burden between WT and Il17d$^{-/-}$ mice in organs such as the heart, lung, and spleen (Fig. S2C).

Phenotype of kidney after i.p. infection of Il17d$^{-/-}$ mice

Our results above suggest the kidney may be an organ site that depends on IL-17D for local immunity. Indeed, *Il17d* but not *Il17a* transcript was induced in the kidney of WT mice infected with GAS at 4 h and 24 h after i.p. injection (Fig. 3D, S2D). This induction led to higher *Ccl2* transcript and CCL2 protein in WT compared to Il17d$^{-/-}$ mice (Fig. 3E, Fig. S2E), suggesting that IL-17D production in the kidney had functional consequences on chemokine production. We found Il17d$^{-/-}$ mice to have slightly decreased overall immune cell in the infected kidney, which coincided with lower Ccl2 protein. Specifically, there was a trend for decreased CD45^{+} cells and neutrophils but not macrophages (Fig. 3F, 3G, 3H) in the kidneys of IL-17D-deficient mice compared to WTs infected with GAS. However, antibody neutralization of CCL2 in WT mice did not lead to higher bacterial burden we saw in Il17d$^{-/-}$ kidney and peritoneum (Fig. S2F, S2G). Furthermore, blocking of CCL2 alone did not compromise immune cell recruitment into the kidney (Fig. S2H, S2I). Since IL-17D can induce chemokines such as *Cxcl3*, *Cxcl2*, and *Ccl7* in vivo (Fig. S1A), we surveyed for transcripts of other downstream effectors that could potentially substitute for CCL2 in WT vs Il17d$^{-/-}$ kidneys (Fig. S2D). Although we found some differences, no single chemokine emerged as clearly responsible for recruiting immune cells in the kidney. In the peritoneum, we found no difference in the absolute numbers of recruited neutrophils and macrophages between WT and Il17d$^{-/-}$ mice (Fig. S2J, Fig. S2K), although we found *Ccl2* transcript was significantly greater in recruited WT peritoneal cells compared to those in *Il17d*$^{-/-}$ mice (Fig. S2L).

Viable, but not non-viable GAS induces IL-17D in non-immune cells

Since the response to GAS differed between locations that had relatively high (peritoneum) versus low (kidney) cellularity in immune cells, we explored the induction of *Il17d* in immune versus non-immune tissues. We performed a short-term *ex vivo* infection of peritoneal exudate cells (PEC) and compared to *in vitro* infection of epithelial cell lines from mouse and human origin. At an MOI of 1:1, GAS challenge was sufficient to elicit increases in *Il17d* transcript in a renal epithelial cell line (HEK293) and PECs (Fig. 4A, Fig. 4B). Induction of *Il17d* in non-immune cells was ablated when the bacteria were heat-killed (Fig. 4A). In contrast, both alive and heat-killed GAS stimulated similar increases in *Il17d* transcript in PECs (immune cells), which are mostly macrophages and B cells (Fig. 4B). Since live bacteria could replicate, we next asked whether the selective induction of IL-17D by live bacteria was a result of increased burden. We found that throughout the time-course of the 3-hour assay, there was an approximately 6-fold increase in live bacteria (Fig. S3A).

Therefore, we titrated the amount of alive or dead bacteria to test whether selective induction of *Il17d* by live versus dead bacteria was due to differences in quantity of bacteria. We found that decreasing the amount of live bacteria 16-fold could still induce *Il17d* better than killed bacteria in epithelial and sarcoma cells (Fig. 4C, S3B), but not in PECs (Fig. 4D). In addition, spiking the assay with additional dead GAS did not increase *Il17d* transcriptional responses in renal epithelial cells, although it was sufficient for commensurate increases in the inflammatory cytokine *Tnf* as a control (Fig. 4E). Similar to dead GAS bacteria, the bacterial cell wall component peptidoglycan did not induce *Il17d*, whereas *Tnf* transcript rose after peptidoglycan treatment or with living or heat-killed GAS (Fig. 4F).

IL-17D induction by viable bacterial requires production of ROS in non-immune cells

We previously found that oxidative stress, via the transcription factor *Nrf2*, could induce IL-17D (16). As viable GAS stimulates oxidative stress through ROS production (37, 36, 42), we next tested whether treating the infected cells with an ROS scavenger attenuated the levels of IL-17D induced. We confirmed that IL-17D is induced in epithelial cells infected by live GAS, while two different ROS scavengers, N-Acetyl-L-cysteine (NAC) and ammonium pyrrolidine dithiocarbamate (PDTC), independently abrogated the effect (Fig. 5A). Notably, *Il17d* induction in immune cells (PECs) were not affected by ROS scavengers, regardless of whether GAS was alive or dead (Fig. S3C). At this early time point, ROS scavengers in the culture medium did not block host cell death induced by GAS cytolytic toxins nor have an inhibitory effect on GAS growth during the experiment (Fig. S3D, Fig. S3E).

In order to test whether ROS scavenger compromises immune responses to bacterial infection, NAC was injected preceding GAS inoculation. We found significantly higher bacterial burden in the kidney but not in the peritoneum in WT mice (Fig. 5B, Fig. 5C). Furthermore, *Il17d* and *Ccl2* transcripts and neutrophil infiltration in the NAC-treated mice were lower compared to control-infected mice (Fig. 5D, Fig. 5E).

Discussion

To apply swift action to fight off pathogens, immune cells recognize pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) leading to effector activities such as production of cytokines (43–46). Most PRRs can respond to dead organisms and even specific molecular components of pathogens. Our data suggest an immune trigger that relies on bacterial viability in addition to PAMPs. Indeed, increasing evidence point to demonstrable distinction between PAMPs produced by the living versus the dead (47–51). By recognizing molecular by-products produced by viable replicating pathogens, the immune response can discriminate between harmless patterns among the normal microbiota and harmful infection (52, 53). Although we have shown that oxidative stress due to viable infection can elicit one specific cytokine (IL-17D), we envision that a more generalizable paradigm could emerge in which other stress pathways, such as metabolic and proteotoxic stress, could also induce cytokine secretion (54) after infection by viable, but not non-viable organisms. Thus, certain immune responses initiate only after the overall “stress” of infection surpasses a threshold. In this way, rather than passively

recognizing a pattern or antigen that the pathogen contains, the host cell must undergo "stress" in order to appropriately respond. Indeed, this paradigm was shown to control the expression of NKG2D ligands, which can be induced by genotoxic stress (55–57).

The PRRs that orchestrate the immune response to GAS are not clearly defined (58–60). Although TLR2 recognizes peptidoglycans found in Gram-positive bacteria (including GAS), it is not required for immune responses to GAS (58). TLR13 in macrophages is required to recognize GAS bacterial RNA in mice (61), and TLR9 recognition of GAS DNA is key to host defense against the pathogen (62). Activation may depend on other components as well, since C-type lectin receptor expressed by macrophages mediates cytokine production by detecting GAS lipoteichoic acid (63).

GAS is known to induce ROS, and oxidative stress caused by invasion-triggered caspases, and ROS production is linked with cell death in macrophages and epithelial cells following GAS infection (37, 36, 42). Attenuated ROS production has been observed in epithelial cells infected by adhesion-incompetent or heat-killed GAS (36, 37, 64). We have previously reported that oxidative stress can lead to IL-17D expression via the activation of the antioxidant transcription factor, Nrf2 (16, 17). Altogether our studies suggest that viable GAS infection induces ROS, leading to oxidative stress and Nrf2 induction, which results in secretion of IL-17D and innate immune responses.

Given that Nrf2 responds to oxidative stress and is critical in protecting cells against ROS-mediated damage, the role of Nrf2 in bacterial immunity is complex (65). For example, in a sepsis model, Nrf2 deficiency results in far higher mortality rate, sensitivity to inflammation by LPS and TNF, and induction of pro-inflammatory genes (66). In addition to the global changes to the immunoreactivity of $Nrf2^{-/-}$ mice, Nrf2-deficient alveolar macrophages are compromised in phagocytic uptake of microbes such as *Pseudomonas aeruginosa* (67). On the other hand, Gomez and colleagues report that mice lacking Nrf2 had increased phagocytic activity at baseline but had compromised immune activation and increased mortality after lung infection by *Streptococcus pneumoniae* (68). It is not clear whether this increased mortality is due to insufficient cell-protective anti-oxidant responses or the lack of IL-17D induction. Although clearly Nrf2 is important for immunity, further studies are needed to clarify the impact of IL-17D in Nrf2-mediated responses.

We demonstrated that $Il17d^{-/-}$ mice are more prone to increased death and weight loss upon opportunistic GAS infection, although with a modest presentation. In addition, the loss of IL-17D resulted in phenotypes limited to some organs such as the kidney, but not in organs such as the spleen and lung. Cytokines in the IL-17 family induces effector molecules that are generally overlapping in similar targets (4, 5, 3). So far, it is known that IL-17A, IL-17F, IL-17E, and IL-17C share at least one receptor subunit, IL-17RA, for signal transduction (2). We speculate that IL-17D is dispensable in spleen and lung because IL-17A is sufficient to confer protection in those organs. In fact, lacking IL-17A in the lung and spleen has been shown to delay and limit neutrophil recruitment after infection by pathogens (69, 70). Furthermore, although IL-17RA and IL-17RC (receptors for IL-17A/F) are protective in candidiasis, IL-17RE (IL-17C cognate receptor) does not confer protection (71). These

observations suggest that some IL-17 cytokines are redundant in tissues with overlapping expression patterns.

Similar to IL-17D, IL-17A promotes neutrophil migration and is generally known to participate in anti-pathogen immunity. However, IL-17A/F and IL-17C promote LPS-induced toxic shock, while IL-17D does not (72–74). Therefore, in addition to overlapping functions of IL-17 cytokines, the degree of inflammation promoted by each may differ by scale. In light of these previous findings, we speculate that the modest effect of IL-17D is attributable in part to the complex overlaps in downstream effectors amongst pro-inflammatory cytokines and especially those within the IL-17 family.

We measured increases in *Il17d* and *Ccl2* upon *in vivo* GAS infection of the WT kidney and also detected a trend for lower neutrophil infiltration and relatively lower *Ccl2* transcript and protein in the kidneys of Il17d$^{-/-}$ compared to WT mice. Since blocking Ccl2 protein did not compromise infiltration or bacterial clearance we surmise that IL-17D can induce other chemokines that work together to recruit the appropriate immune response, perhaps redundantly. Indeed, recombinant IL-17D induced a plethora of chemokines including Cxcl3, Ccl7 and Ccl5. We speculate that the fact that the kidney harbors a relatively low ratio of immune cells to non-immune cells when compared to organs such as lungs and spleen explains its reliance on IL-17D-induced chemokines for anti-pathogen immunity. Thus, Il17d$^{-/-}$ mice may face greatest disadvantage in warding off bacterial infection in organs dependent on effective recruitment of early active responders. Notably, unregulated IL-17D production in locations that have low levels of resident immune cell could lead to pathology. Therefore, it is logical to place a checkpoint in non-immune cells limiting their production of IL-17D only during live infection, as we have shown. This live/dead checkpoint could serve as a paradigm to regulate immune responses in tissues with low levels of immunity but that may also harbor commensal organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key point summary

1. GAS infection in Il17d$^{-/-}$ mice leads to greater bacterial burden in kidneys.
2. Viable, but not heat-killed, GAS induces Il17d in non-immune cells.
3. A scavenger of reactive oxygen species inhibits Il17d induction in non-immune cells.

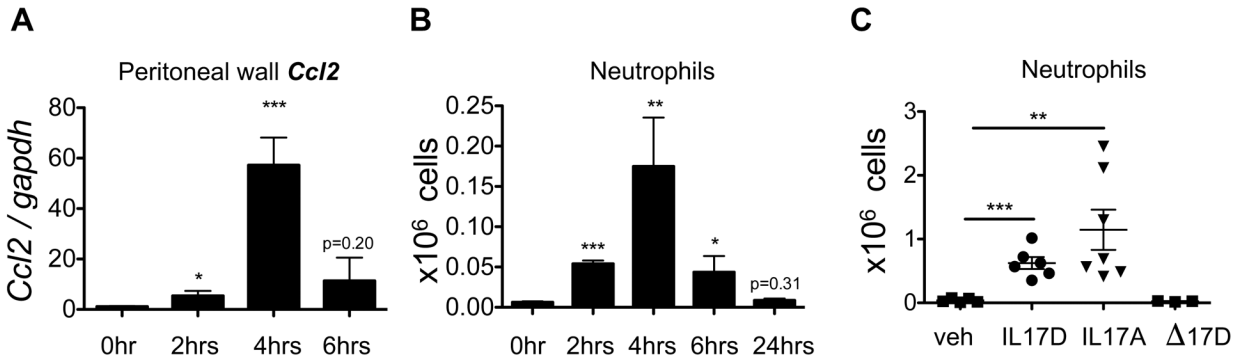

Figure 1. Recombinant IL-17D induces Ccl2 and recruits innate immune cells into the peritoneum.

(**A**) Relative *Ccl2* transcript in peritoneal wall tissue (mesothelial cells) hours after i.p. IL-17D injection (n=4). (**B**) Total peritoneal exudate neutrophils (n=4) recovered hours after i.p. IL-17D injection. (**C**) Neutrophils recovered in peritoneal exudate after 4h i.p. IL-17D versus IL-17A (n=3). Data are combined from 2 experiments.

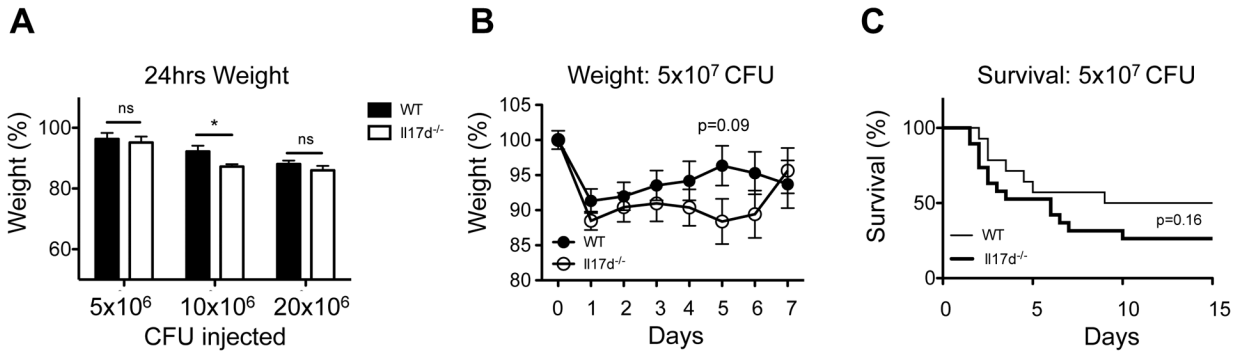

Figure 2. GAS-infected Il17d$^{-/-}$ mice have slight weight loss and a decreased survival rate compared to WT mice.

(**A**) Percent weight loss of WT and Il17d$^{-/-}$ mice (n=3) after 5×10^6, 1×10^7, and 2×10^7 i.p. GAS doses. (**B**) Percent weight loss and (**C**) percent survival of WT (n>6) versus *Il17d−/−* (n>8) mice after 5×10^7 dose of GAS i.p. Data are combined from at least 2 independent experiments. The p value in (**B**) corresponds to the Day5 timepoint.

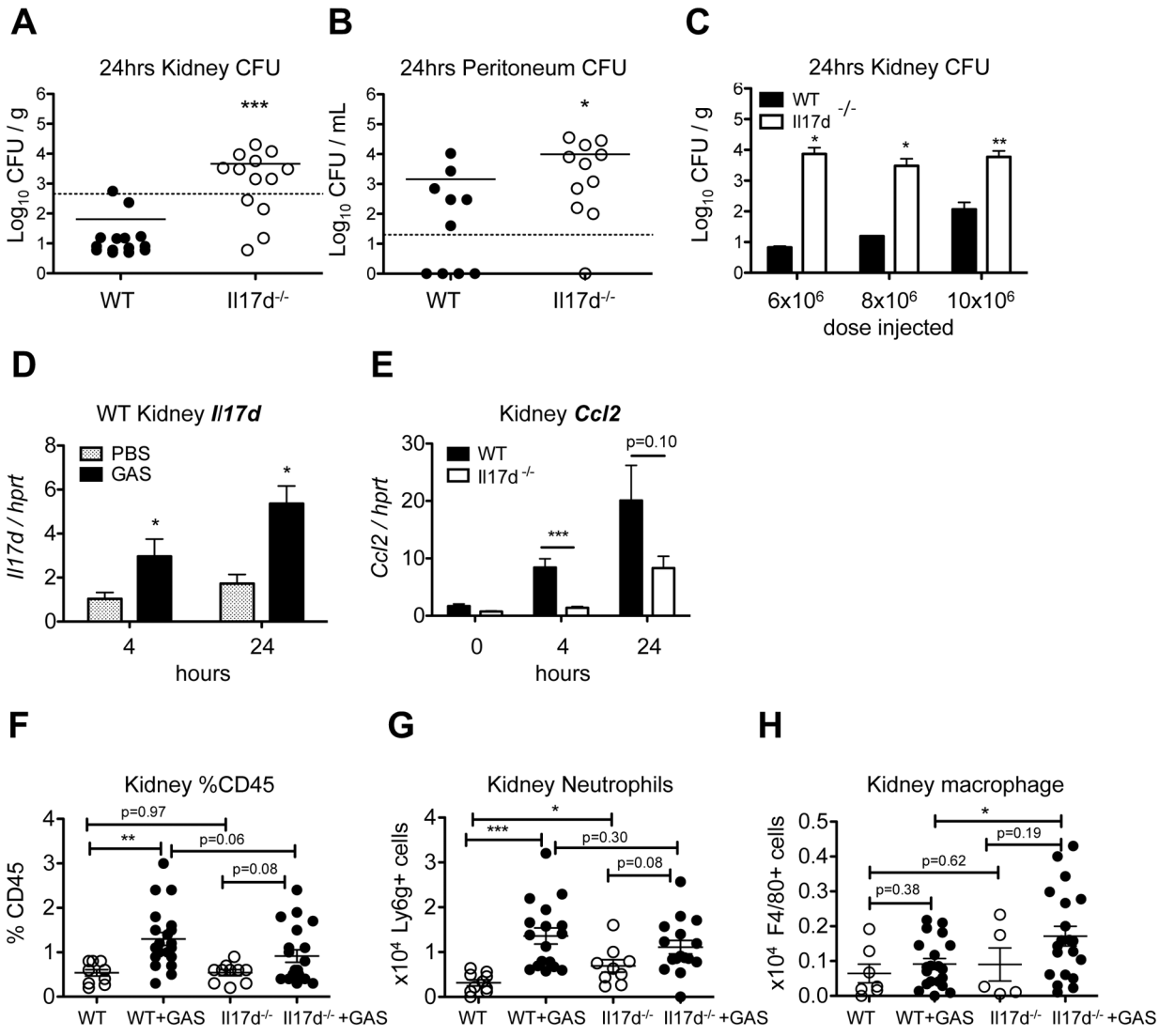

Figure 3. IP injection of GAS into Il17d$^{-/-}$ mice results in increased bacterial burden in kidney.

Bacterial CFU burden in (**A**) kidney homogenates and (**B**) peritoneal cavity at 24 hours after GAS infection (dose=10×10^6) in WT and Il17d$^{-/-}$ mice (n>5). (**C**) CFU recovered in kidney (n=3) after 24 hours of three doses (6×10^6, 8×10^6, 10×10^6) of GAS i.p. (**D**) Relative ex vivo *Il17d* transcript in the GAS-infected kidney of WT mice compared to PBS control injection group (n=4). (**E**) Relative *Ccl2* transcript of WT and Il17d$^{-/-}$ kidney from GAS-infected mice (n=3–5). (**F**) Percentage of CD45$^+$ cells and absolute (**G**) neutrophil and (**H**) macrophage numbers in the kidney from uninfected (n=2–4) and 24 hours post-infected (n=4–6) WT and Il17d$^{-/-}$ mice. Data are combined data of at least 2 independent experiments.

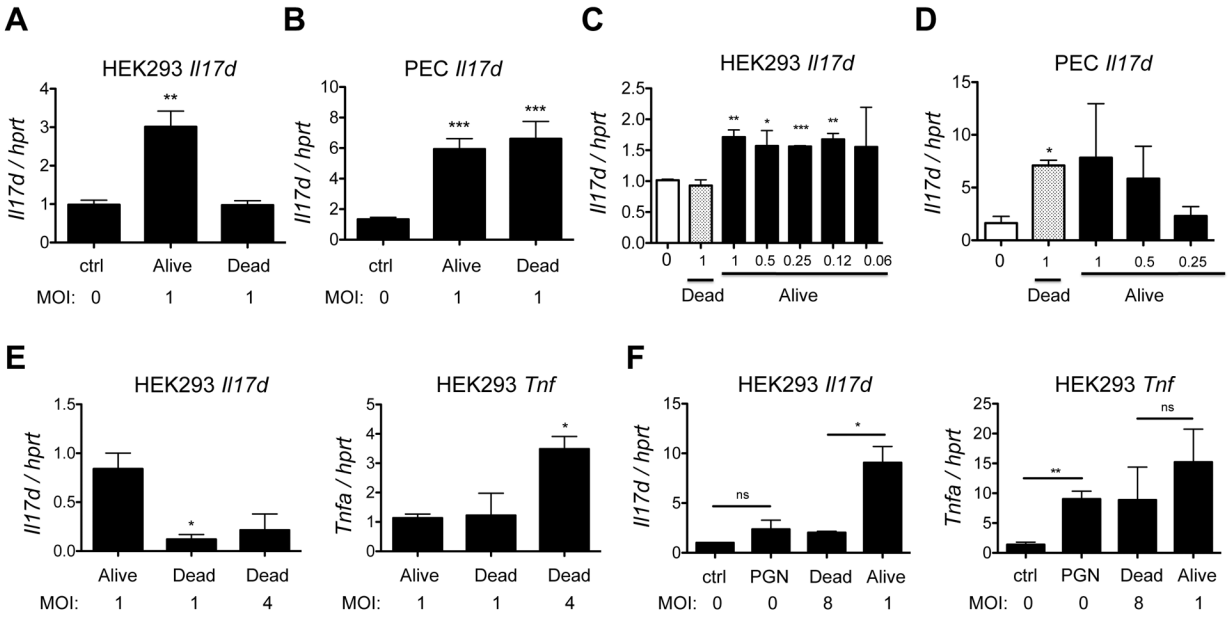

Figure 4. Viable GAS induces IL-17D in non-immune cells.

(**A**) Relative *Il17d* transcript in HEK293 cells and (**B**) peritoneal exudate cells (PECs) after 2-hour in vitro incubation with the indicated MOI (multiplicity of infection) using living (alive) or heat-killed (dead) GAS (n=3). (**C**) Relative *Il17d* transcript in the HEK293 and (**D**) PECs after 3-hour incubation with decreasing GAS infection ratio (max MOI of dead and alive is 1:1) (n=3). (**E**) Relative *Il17d* and *Tnf* in HEK293 cells incubated with increasing ratio of heat-killed GAS (n=3). (**F**) HEK293 cells were incubated with peptidoglycan (PGN), dead GAS (8:1), and living GAS (1:1) and transcript levels of *Il17d* and *Tnf* were measured (n=3). Data are representative data of at least 3 independent experiments.

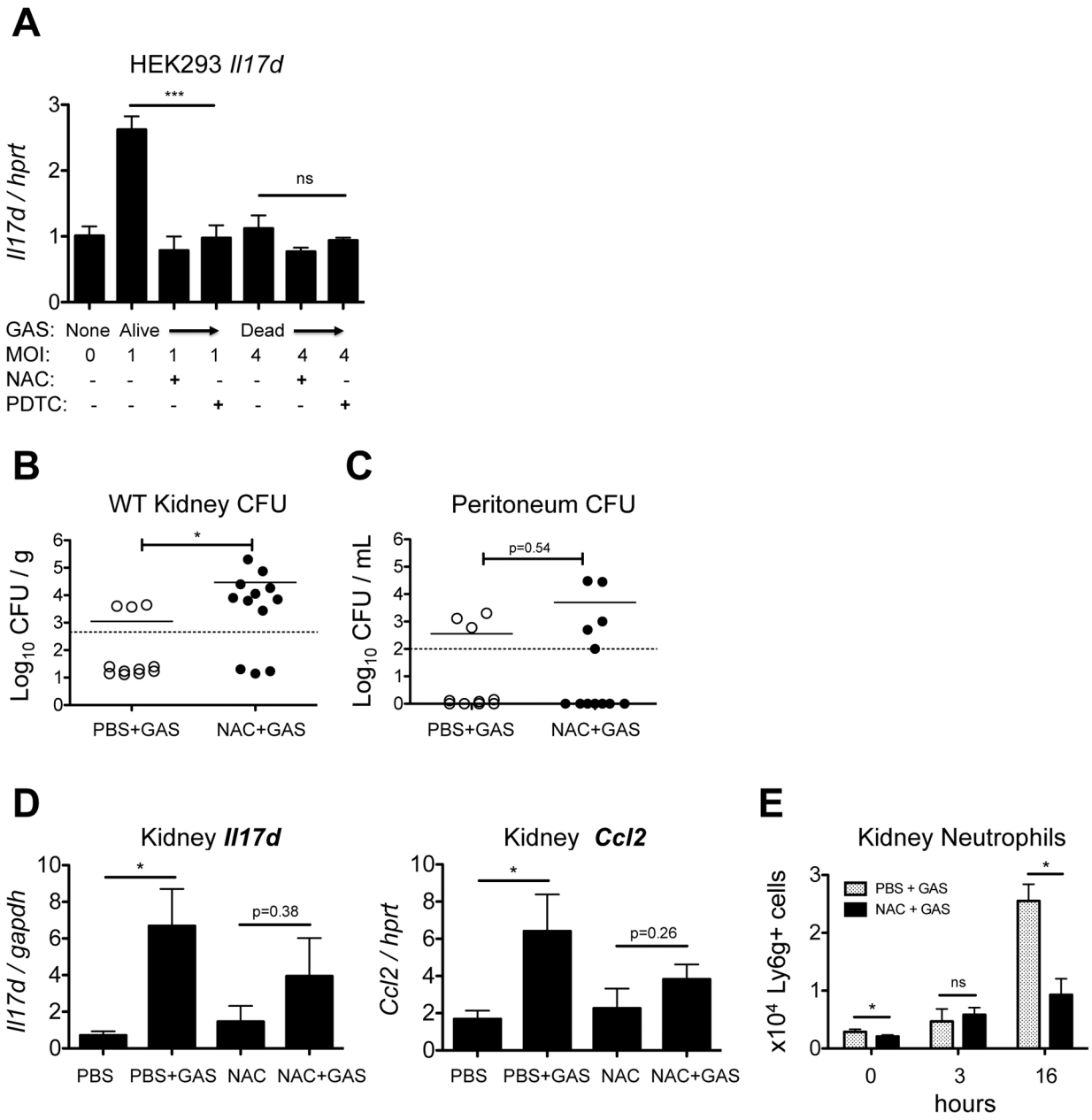

Figure 5. ROS scavenger suppresses induction of IL-17D in active GAS infection.

(**A**) Relative *Il17d* transcript in the HEK293 cells (n=3) pre-treated with ROS scavengers (NAC and PDTC) prior to incubating with GAS (1:1 alive; 4:1 dead). (**B**) Kidney and (**C**) peritoneal cavity bacterial CFU burden after 16h from GAS-infected and ROS scavenger-treated mice (n>4). (**D**) Relative kidney *Il17d* and *Ccl2* transcript in infected mice pre-treated with ROS scavenger (n=3). (**E**) Absolute numbers of kidney neutrophils in GAS-infected mice with or without ROS scavenger (n>3). Data are combined data of at least 2 independent experiments.