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TRANSLATIONAL SCIENCE

Card9/neutrophil signalling axis promotes IL-17A-mediated ankylosing spondylitis

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ABSTRACT

Objective Polymorphisms in the antifungal signalling molecule *CARD9* are associated with ankylosing spondylitis (AS). Here, we investigated the cellular mechanism by which CARD9 controls pathogenic Th17 responses and the onset of disease in both experimental murine AS and patients.

Methods Experiments in SKG, Card9$^{-/-}$SKG, neutrophil-deplete SKG mice along with *in vitro* murine, neutrophil and CD4^{+} T cell cocultures examined Card9 function in neutrophil activation, Th17 induction and arthritis in experimental AS. In AS patients the neutrophil: Bath Ankylosing Spondylitis Functional Index relationship was analysed. *In vitro* studies with autologous neutrophil: T cell cocultures examined endogenous CARD9 versus the AS-associated variant (rs4075515) of CARD9 in T cellular production of IL-17A.

Results Card9 functioned downstream of Dectin-1 and was essential for induction of Th17 cells, arthritis and spondylitis in SKG mice. Card9 expression within T cells was dispensable for arthritis onset in SKG mice. Rather, Card9 expression controlled neutrophil function; and neutrophils in turn, were responsible for triggering Th17 expansion and disease in SKG mice. Mechanistically, cocultures of zymosan prestimulated neutrophils and SKG T cells revealed a direct cellular function for Card9 within neutrophils in the potentiation of IL-17 production by CD4^{+} T cells on TCR-ligation. The clinical relevance of the neutrophil-Card9-coupled mechanism in Th17-mediated disease is supported by a similar observation in AS patients. Neutrophils from HLA-B27^{+} AS patients expanded autologous Th17 cells *in vitro*, and the AS-associated CARD9^{S12N} variant increased IL-17A.

Conclusions These data reveal a novel neutrophil-intrinsic role for Card9 in arthritogenic Th17 responses and AS pathogenesis. These data provide valuable utility in our future understanding of CARD9-specific mechanisms in spondyloarthritis .

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ CARD9 is a fundamental signalling-molecule in Th17-responses and antifungal immunity; thereby positioning it at a compelling juncture between host defence, the Th17 cells and ankylosing spondylitis (AS).
⇒ CARD9 and Th17/IL-17A responses have been independently implicated in AS pathogenesis.

WHAT THIS STUDY ADDS

⇒ Card9-signalling promoted AS-like disease in genetically susceptible SKG mice.
⇒ Card9 expression within neutrophils is critical and sufficient to induce Th17 cell expansion and neutrophils were essential to Th17 induction and onset of experimental AS.
⇒ In AS patients, neutrophils were positively associated with increased disease severity and augmented in recently diagnosed (<5 years) patients.
⇒ AS patient neutrophils were alone sufficient to enhance autologous Th17 responses, cumulatively supporting a critical role for neutrophils in direct control of pathogenic IL-17 responses in AS.
⇒ AS risk variant CARD9^{S12N} was associated with increased plasma IL-17A in AS.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Activation of the Card9/neutrophil axis promotes the pathogenesis of AS by enhancing the pathogenic Th17 responses and the CARD9^{S12N} variant is directly associated with increased IL-17A. These data will contribute to our understanding of pathogenic cellular mechanisms mediated specifically by the CARD9/Th17-pathway in AS patients, thereby directing more optimal usage of anti-Th17 therapy and potentially novel neutrophil targets.

INTRODUCTION

Ankylosing spondylitis (AS), also referred to as axial spondyloarthritis (SpA), is a severe form of inflammatory arthritis that targets the spine resulting in chronic pain and vertebrae fusion (termed 'bamboo spine'). AS is part of a clinically diverse group of rheumatological diseases termed 'SpA' that primarily affect the peripheral joints, entheses and spine, but can also be associated with inflammation in other organ systems such as the eye, skin, lung and intestine. SpAs have overlapping characteristics in terms of genetics and symptoms, wherein AS is the most prevalent.[1 2] The high predominance of human leucocyte antigen B27 (HLA-B27) positivity (>90%) in AS patients, along with other HLA-associated variants, supports the relationship between HLA: cognate antigen and self-reactive (ie, autoreactive) T cells.[3 4] In particular, CD4^{+} T helper

cells that produce IL-17 (Th17 cells) drive the pathogenesis of AS,[5–7] with IL-17 being a current therapeutic target for disease.[7] Although IL-17A blockade has been greatly effective, remission is achieved in only a portion of AS patients (~65.5%).[8] These data suggest that AS pathogenesis can be driven by multiple disease mechanisms including those that are Th17-dependent and those that are Th17-independent.

While historically considered autoimmune (ie, self-reactive T cells and/or autoantibodies) an emerging alternative concept supports AS being a 'mixed-pattern' condition,[9] which features both autoimmune and autoinflammatory (ie, hyperinnate) responses that are autoantigen-independent. Yet, exactly how innate cellular reactions interact with autoreactive T cells to trigger AS is largely unknown. Hence, understanding how minor alleles (ie, non-HLA-B27) and non-genetic factors such as environmental triggers collaboratively shape disease would be of considerable interest and importance to human health.

Outside of HLA-B27, caspase recruitment domain-containing protein 9 (*CARD9*) variants confer significant risk to AS.[5 10–12] One of the more common variants is the rs4077515 allele A that results in amino acid substitution of asparagine at position 12 for serine ($CARD9^{S12N}$).[11 13] Card9 is a key adaptor protein responsible for C-type lectin receptor (CLR)-mediated host defence against fungal and pathogenic mycobacterial infections.[14] After microbial engagement of CLRs (eg, Dectin-1, Dectin-2, Mincle), Card9 couples with Bcl10 and Malt1, and activates the canonical downstream signalling via the kinase ERK and the transcription factor NF-κB, to facilitate Th17-immunity.[14] This places Card9 at a compelling juncture between environmental triggers, the Th17 pathway and AS. Card9 is highly conserved between mice and humans[14] where Card9-deficiency in mice,[15] or loss-of-function mutations in humans, result in invasive fungal infection and immunodeficiency disorders.[14 16 17] However, any mechanistic function of CARD9 in AS has remained to be determined.

The goal of our study was to determine how Card9 affects upstream or downstream pathways that potentiate arthritogenic Th17 cells and AS pathogenesis. To do this, we employed the monogenetic model of AS in SKG mice.[18–21] SKG mice have a point mutation in the T cell receptor signalling molecule $Zap70^{W163C}$, which diminishes the TCR-signalling threshold.[22] This in turn, impairs negative selection in the thymus[22] and results in generation of autoreactive $CD4^+$ T cells. Th17 cells are enriched in the peripheral T cell repertoire and are necessary for induction of arthritis.[23] Thus, akin to how AS is viewed, disease in SKG mice represents a Th17-dependent disease that stems from a break in central tolerance. We and others have shown that disease manifestations in SKG mice resemble the spectrum of SpA, including AS-like pathology within the enthesitis and lower vertebrae,[18–21] as well as periocular disease,[18] psoriatic skin lesions,[21] interstitial lung disease[24] and enteritis/ileitis.[18 19 25] Importantly, the full spectrum of disease is influenced by the environment, with fungal-derived β-glucan-containing products such as zymosan[26] being especially potent triggers.

Using genetically susceptible SKG mice and samples from AS patients, our data reveal a novel mechanism by which environmental fungi trigger Dectin-1 on neutrophils through a Card9-mediated mechanism to subsequently induce arthritogenic Th17 cell expansion and onset of arthritis. These data shape how we understand CARD9-mediated disease in AS patients and contribute to our understanding of the genesis of pathogenic Th17-mediated responses in AS.

METHODS

Animals

We used the ARRIVE1 reporting guidelines.[27] Detailed methods are described in online supplemental methods section.

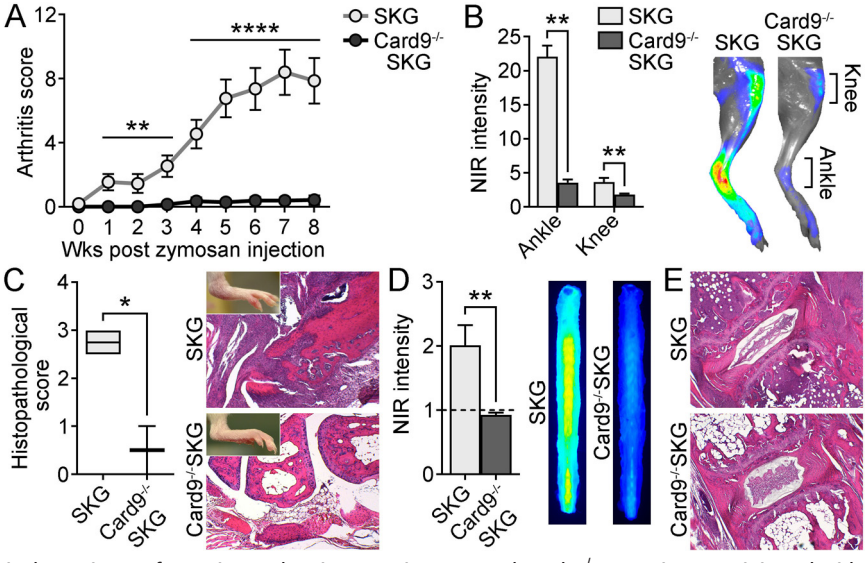

Figure 1 Card9 is a genetic determinant of experimental AS in SKG mice. SKG and $Card9^{-/-}$SKG mice were injected with zymosan. (A) Mice were scored weekly for clinical arthritis. Data are combined from two experiments, n=11–14 total mice/group. (B) Visualisation and quantification of NIR signal within the legs. Regions of interest (ROIs) were quantified and mean differences between ROIs were normalised to those of corresponding ROI of healthy (non-arthritic), naïve WT-BALB/c mice. (C) Histopathology of the ankles at 8 weeks post-zymosan with corresponding photographical representation of ankles (inset). Data are combined from two experiments, n=11–14 total mice/group. (D) NIR quantification of the spine and (E) corresponding representative histopathology at 8 weeks post-zymosan. Data are mean±SEM, except for C, which shows medians with the 25th and 75th IQR (box) and min and max (whisker). Data were analysed with unpaired, two-tailed Student's t-test or Mann-Whitney U test (C), *p<0.05, **p<0.01, ****p<0.0001. AS, ankylosing spondylitis; NIR, near-infrared.

Induction and clinical and histological assessment of arthritis
Details for induction of arthritis, and assessments of arthritis are detailed in online supplemental file.

Near-infrared *in vivo* imaging
Imaging of dissected legs and spines has been described previously[18] using the protease substrate, ProSense (NEV10003, Perkin Elmer).

Intracellular cytokine staining and flow cytometry on mouse and human cells
Detailed methods are described in online supplemental methods section.

Peritoneal lavage protocol and ELISA cytokine analysis
Detailed methods are described in online supplemental methods section.

Thymocyte transfer
Single-cell suspensions were prepared from thymi of naïve donor SKG or Card9$^{-/-}$SKG mice. Purified thymocytes (1×10^8) were intravenous injected into sex-matched Rag1$^{-/-}$ recipients who were then i.p. injected with zymosan 24 hours later.

Murine and AS patient ex vivo neutrophil: CD4$^+$ T cell cocultures
Detailed methods are described in online supplemental methods section.

In vivo neutrophil depletion
Mice were i.p. injected with 300 µg/200 µL anti-Ly6C mAb (clone 1A8; Bio X Cell) on days −1, 0, 2, 4 relative to zymosan injection. Our own data have confirmed >90% reduction in circulating neutrophils over a 3-dy period, as determined by flow cytometry.

Statistics for animal studies
Detailed methods are described in online supplemental methods section.

Human subject population information, variables and statistical analysis
Detailed methods are described in online supplemental methods section.

AS patient DNA purification and CARD9^{S12N} genotyping
Detailed methods are described in online supplemental methods section.

Human serum IL-17A ELISA
Detailed methods are described in online supplemental methods section.

Patient and public involvement statement
Patients were not involved in the study as the study was done on their circulating immune cells.

RESULTS
The Dectin-1/Card9-coupled signalling axis is responsible for induction of experimental AS in SKG mice
Experimental AS was examined in Card9$^{-/-}$ mice with homozygous expression of Zap70^{W163C} (Card9$^{-/-}$SKG mice). SKG

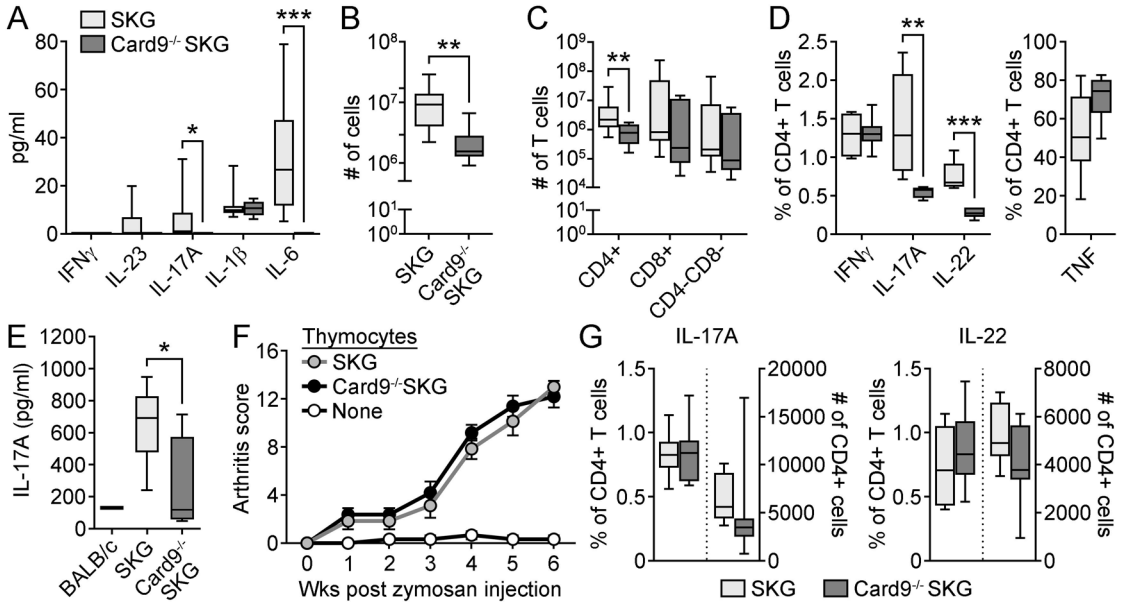

Figure 2 Card9 promotes peripheral Th17 expansion independent of central tolerance mechanisms. (A) Serum cytokines were quantified by ELISA at 8 weeks post-zymosan. Data are combined from two experiments for n=6–11 total mice/group. The total number of live cells (B) and Thy1.2$^+$ T cell subsets (C) in the popliteal draining lymph nodes (dLN) were evaluated by flow cytometry at 8 weeks post-zymosan. (D) The per cent of indicated cytokine$^+$CD4$^+$ T cells in the dLN was evaluated by intracellular cytokine staining and flow cytometry at 8 weeks post-zymosan. For (B–D), data were combined from three experiments, n=13 total mice/group. (E) IL-17A in the synovial fluid of ankles was measured by ELISA at 8weeks post-zymosan in SKG and Card9$^{-/-}$SKG mice or naïve (no zymosan) WT (BALB/c) mice. Data are combined from two experiments, n=5–6 total mice/group. (F) Arthritis scores of Rag1$^{-/-}$ mice reconstituted with 10^8 thymocytes from either naïve SKG or naive Card9$^{-/-}$SKG mice were evaluated weekly post-zymosan. (G) IL-17A and IL-22 expression by CD4$^+$ T cells in dLN of naïve (no zymosan) SKG versus Card9$^{-/-}$SKG mice. For F–G, Data are combined from two experiments, n=6–7 mice/group. Data are shown as medians with the 25th and 75th IQR (box) and min and max (whisker); except for (F) which shows mean±SEM. Data were analysed with Mann-Whitney U test (C), *p<0.05, **p<0.01, ***p<0.001.

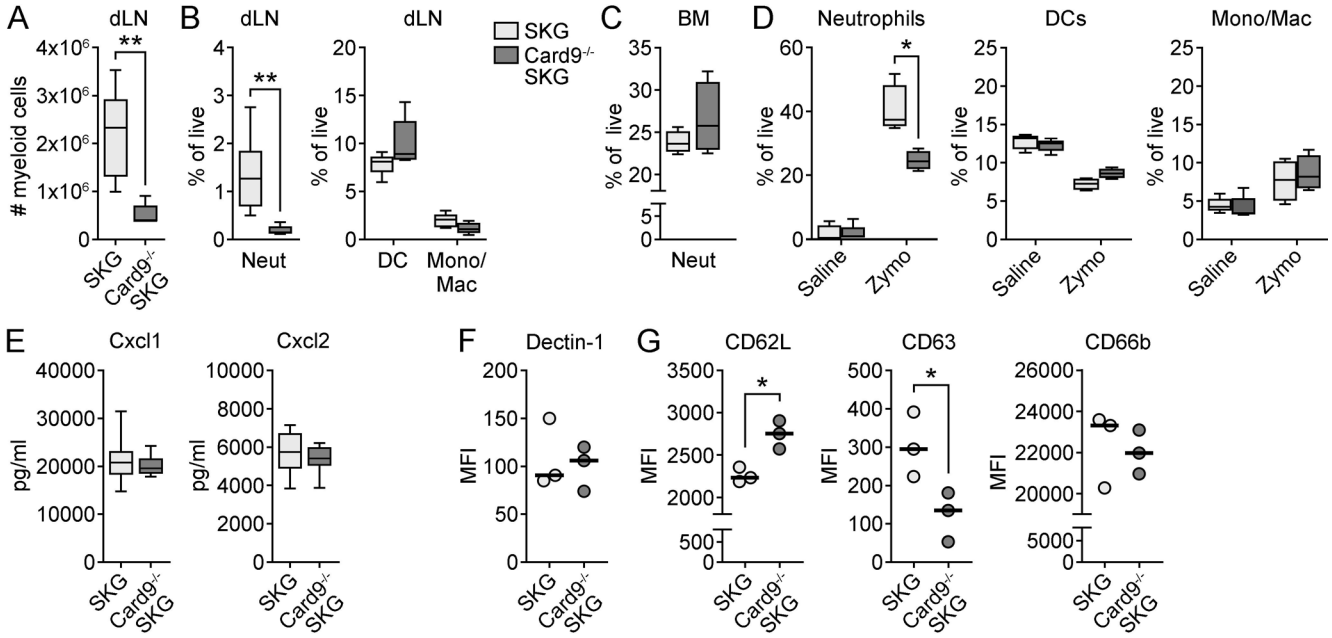

Figure 3 Card9 controls peripheral activation of neutrophils in SKG mice. (A) Total # of myeloid cells (Thy1.2$^-$B220$^-$ that express CD11b, CD11c or Ly6g) in the dLN; and (B) frequency of myeloid subpopulations were evaluated in the dLN at 8 weeks post-zymosan. Neutrophils: CD11b$^+$CD11c$^-$Ly6g$^+$, DCs: CD11b^{variable}CD11c$^+$Ly6g$^-$ and monocyte/macrophages: CD11b$^+$CD11c$^-$Ly6g$^-$. (C) Neutrophil composition in bone marrow (BM) of naïve SKG or naïve Card9$^{-/-}$SKG mice. (D–G) Mice were injected with zymosan or saline and 4 hours later peritoneal lavage fluid was evaluated for (D) myeloid cell composition by flow cytometry and (E) levels of Cxcl1 and Cxcl2 by ELISA. Data are combined from two experiments, n=8–10 mice/group; and are shown as medians with the 25th and 75th IQR (box) and min and max (whisker). Data were analysed with unpaired, two-tailed Student's t-test or Mann-Whitney U test (C), *p<0.05, **p<0.01. (F–G) Median fluorescence intensity (MFI) of indicated cell surface molecules on neutrophils in peritoneal lavage fluid were quantified by flow cytometry, with each dot representing an individual mouse. Representative data of one of three individual experiments (n=3 mice/genotype/experiment).

mice developed progressive inflammatory arthritis within the ankles and knees, whereas Card9$^{-/-}$SKG mice showed little, to no signs of clinical disease (figure 1A). Using near-infrared (NIR)-imaging, we further quantified localised joint inflammation was ameliorated in Card9$^{-/-}$SKG mice (figure 1B). These clinical and molecular changes were corroborated by histopathology within the ankles, which revealed severe synovitis, massive sub synovial cell infiltrate, pannus formation and erosion of adjacent cartilage and subchondral bone in SKG mice (figure 1C).

Our prior NIR-imaging of disease in SKG mice revealed that axial inflammation localised to the lower lumbar spine and upper caudal vertebrae of the tail.[18] Examination of this specific spinal region by NIR-imaging and histology showed reduced NIR-signal (figure 1D) and reduced inflammation within the spinal joints (figure 1E), including inflammation within dissected lower lumbar vertebrae; and vertebral tendon and ligaments, inflammatory infiltrate within the paravertebrae space, vertebral erosion and disc compression. Collectively, these data support a function for Card9 in promoting experimental AS.

The fungal receptor Dectin-1 has been shown to be essential in the induction of arthritis in SKG mice, in that Dectin-1 blockade impaired zymosan-triggered arthritis.[26] Since Card9-mediates downstream Dectin-1 signalling, this implicates a Dectin-1/Card9-coupled mechanism in SKG mice. However, additional CLR-agonists have also been reported to activate the Card9-signalling pathway in lieu of zymosan or Dectin-1. Thus, we considered whether Card9 served as an integral signalling mediator downstream of additional CLRs such as Dectin-2 and Mincle that are vital for control of both *Candida albicans*[15] and *Mycobacterium tuberculosis* (Mtb) infections.[28] Mtb is a key constituent of complete Freund's adjuvant (CFA), where Mincle activation is important for induction of experimental autoimmunity, including experimental autoimmune uveitis, collagen-induced arthritis and experimental autoimmune encephalomyelitis.[29 30] Somewhat surprisingly Mtb components, Dectin-2 or Mincle triggers were incapable of inducing arthritis in SKG mice (online supplemental figure 1). However, additional Dectin-1 specific triggers such as Heat killed *C. albicans* or β−1,3-glucan curdlan were capable of triggering arthritis akin to zymosan via a Card9-dependent mechanism. These data indicate a direct Dectin-1/Card9-coupled signalling mechanism in experimental AS.

Card9 promotes peripheral Th17 expansion independent of central tolerance mechanisms

We next evaluated how Card9 affects Th17-responses in SKG mice. Plasma levels of IL-17A and IL-6 were significantly reduced in Card9$^{-/-}$SKG compared with SKG mice (figure 2A). Total cellularity of the popliteal lymph nodes, (ie, the relevant draining lymph nodes (dLN) of the affected leg joints) was reduced in Card9$^{-/-}$SKG mice (figure 2B), which was attributed to a significant decrease in CD4$^+$ T cells (figure 2C). In terms of function, CD4$^+$ T cells in Card9$^{-/-}$SKG mice produced less Th17-associated cytokines IL-17A and IL-22 (figure 2D) compared with SKG mice, whereas production of the Th1-associated cytokines, TNF and IFNγ, were similar in both strains of mice. Within the ankle, IL-17A levels in the synovial fluid of Card9$^{-/-}$SKG mice were significantly reduced (figure 2E). Cumulatively, these findings indicate a role for Card9 in control over Th17-mediated disease in SKG mice.

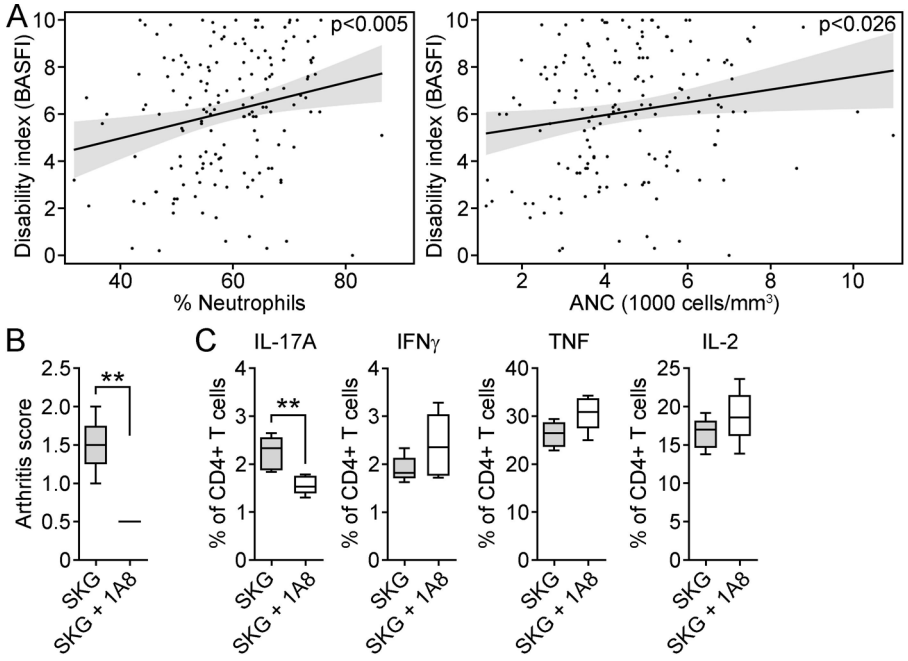

Figure 4 Neutrophils relate to increased AS disease burden and are necessary for induction of Th17 cells and arthritis in SKG mice. (A) A linear regression analysis was conducted with only the patients from online supplemental table 1, that were biologic-naïve and diagnosed with AS, to compare the disability index (BASFI score) with either the per cent of neutrophils or the absolute neutrophil count (ANC) in the blood. Data are displayed with best-fit line and 95% CIs for n=158 longitudinal laboratory observations, clustering of 72 patients. Per cent of neutrophil data correlation coefficient 0.059, p=0.005, cluster analysis (clustering 72 patients): correlation coefficient unchanged, p=0.042. ANC data correlation coefficient was 0.274, p=0.026, cluster analysis (clustering 72 patients); correlation coefficient unchanged, p=0.077. (B, C) SKG mice or SKG mice deplete of neutrophils via 1A8 mAb (anti-Ly6G) were injected with zymosan and 5 days later were evaluated for: (B) arthritis and (C) the percent of indicated cytokine+CD4$^+$ T cells in the dLN by flow cytometry. Data are shown as medians with the 25th and 75th IQR (box) and min and max (whisker), and were analysed with Mann-Whitney U test, *p<0.05, **p<0.01. Shown is a representative experiment of three independent repeats with n=3–5 mice/condition/experiment. AS, ankylosing spondylitis; BASFI, Bath Ankylosing Spondylitis Functional Index; dLN, draining lymph nodes.

A current paradigm supports a break in central tolerance as the underlying cause of autoimmunity based on dysregulated HLA-B27 function,[31] and it is also the prevailing mechanism of disease within SKG mice (although via the Zap70^{W163C} mutation).[22] Thus, we evaluated a role for Card9 within the thymus in development of T cells and induction of arthritis. Equal numbers of thymocytes from naïve (non-arthritic) SKG or Card9$^{-/-}$SKG mice were adoptively transferred into lymphopenic Rag1$^{-/-}$ recipients and arthritis was evaluated following zymosan (figure 2F). Card9$^{-/-}$SKG and SKG thymocytes were equally capable of triggering arthritis, indicating normal thymocyte development in Card9$^{-/-}$SKG mice; and that the cellular mechanism by which Card9 induces arthritis must be independent of T cell development or central tolerance.

In SKG mice, precommitted arthritogenic T cells escape the thymus and populate the periphery where they spontaneously differentiate into Th17 cells in naïve SKG mice.[23] Thus, we considered a role for Card9 within the periphery in spontaneous differentiation of autoreactive Th17 cells in SKG mice. We found that peripheral expansion of the Th17 population, as identified by IL-17A and IL-22 production, was unchanged in naïve Card9$^{-/-}$SKG versus SKG mice (figure 2G). This observation is in line with other reports that Card9 is dispensable for T cell development and peripheral TCR-mediated activation in healthy, wild-type strains of mice.[28 32] An important aspect of the SKG model is that despite the spontaneous expansion of arthritogenic Th17 cells in naïve SKG mice, they do not develop the full spectrum of disease (ie, arthritis or spondylitis) until they are challenged with microbialβ-glucans. Collectively our data, support a T cellular-extrinsic function for Card9 within the periphery in the expansion of Th17 cells on Dectin-1 activation.

Card9 controls peripheral activation of neutrophils in SKG mice

Card9 is predominately expressed in myeloid cells including macrophages, DCs and neutrophils.[32 33] An *in vivo* contribution of myeloid cells in the induction of arthritis in SKG mice is supported by studies that show that treatment with either chlodronate liposomes or Gr-1 mAb (ie, targets both Ly6G$^+$ neutrophils and Ly6C$^+$monocytes)[34] impairs onset of arthritis.[35 36] Thus, we sought to examine putative perturbations in myeloid cell responses in arthritic SKG versus Card9$^{-/-}$SKG mice. Total myeloid cellular responses were greatly reduced in the dLN of Card9$^{-/-}$SKG mice versus SKG mice (figure 3A). Importantly, reduced myeloid cellularity in Card9$^{-/-}$SKG mice appeared to be due to reduced neutrophil-specific responses, whereas macrophage or DC responses were unaltered in Card9$^{-/-}$SKG versus SKG mice (figure 3B).

As rapid responders to β-glucans, neutrophils quickly undergo changes in their activation status and functions. Thus, we sought to further evaluate how Card9 controls acute neutrophil responsiveness. Importantly, neutrophil development within the bone marrow in naïve mice was unaltered by Card9-deficiency (figure 3C). However, 4 hours post-zymosan injection, we observed marked neutrophilia in the peritoneal cavity of SKG mice, which was significantly reduced in Card9$^{-/-}$SKG mice (figure 3D). We did not observe any genotype-specific differences in macrophage or DC populations between the two strains of mice (figure 3D). Since a Card9-mediated mechanism involving Cxcl1 and Cxcl2 has been established in neutrophil recruitment during *C. albicans* infection and experimental

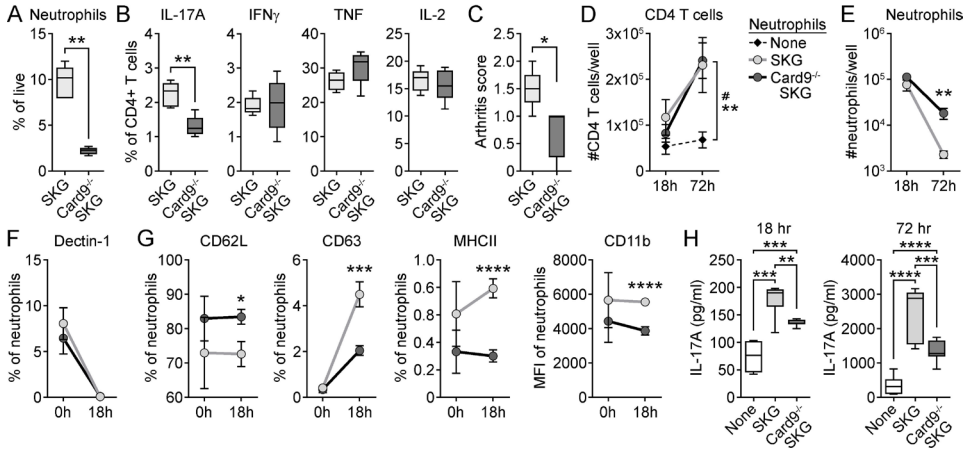

Figure 5 Card9-signalling within neutrophils promotes their activation and ability to expand Th17 cells. (A) The dLNs of mice were evaluated 5 days after zymosan injection by flow cytometry for the percentage of (A) neutrophils and (B) cytokine$^+$ CD4$^+$ T cells. (C) Early signs of arthritis were measured 5 days post-zymosan. Data shown are representative experiment of three independent repeats with n=5 mice/genotype/experiment. (D–H) Neutrophils purified from naïve SKG or naïve Card9$^{-/-}$SKG mice were stimulated with zymosan for 1 hour, washed, and then cocultured with SKG CD4+T cells (two neutrophils: 1 T cell) in the presence of mAb to CD3 and CD28 for 18 hours or 72 hours. (D) at 72 hours post-coculture, the total number of SKG CD4+T cells in each culture condition was quantified by flow cytometry. $^{\#}$p=0.03, comparison between SKG (SKG neutrophils: SKG T cells) versus none (SKG T cells only), and **p=0.001, comparison between Card9$^{-/-}$SKG (Card9$^{-/-}$–SKG neutrophils: SKG T cells) versus none. (E) The total number of neutrophils/well and (F–G) cell surface expression of indicated markers was quantified on neutrophils freshly isolated (pre-zymosan stimulation and pre-coculture) from naïve SKG or Card9$^{-/-}$–SKG mice (0 hour) or 18 hours post coculture by flow cytometry. (H) IL-17A was measured in culture supernatants at 18 hours and 72 hours post-coculture by ELISA. Data were analysed with Mann-Whitney U test (A–C) or unpaired, two-tailed Student's t-test (D–H). *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001, cumulative data from three independent experiments, 3–4 biological replicates/condition/experiment. dLN, draining lymph nodes.

autoantibody-induced arthritis,[37 38] we measured Cxcl1 and Cxcl2 levels in peritoneal lavage fluid. However, we did not observe any genotypic differences (figure 3E).

We next evaluated how Card9 controls neutrophil activation in response to zymosan in SKG mice. Although Dectin-1 is expressed by all myeloid cells, neutrophils specifically, have been shown to depend on Dectin-1 for recognition and cellular responses to fungal particles.[39] We found that cell surface expression of Dectin-1 on neutrophils 4 hours post-zymosan to be comparable for both genotypes (figure 3F), indicating that neutrophils from Card9$^{-/-}$SKG mice retain the ability to properly senseβ-glucans. Nonetheless, the activation status and function of Card9$^{-/-}$SKG neutrophils was significantly impaired compared with SKG neutrophils, as indicated by retention of CD62L and a concomitant decrease in exocytosis of primary and secondary granules, indicated by decreased CD63 and CD66b expression, respectively (figure 3G). Collectively, these data reveal an important function for Card9 in controlling acute, neutrophil responsive to Dectin-1 activation within in SKG mice.

BASFI=functional index (how bad of damage−disease severity) accounts for BASDI.

BASDI is a moment in time disability index (based on series of questions).

Neutrophils relate to increased AS disease severity and are necessary for induction of Th17 cells and arthritis in SKG mice

Our data indicate a critical function for Card9 in controlling neutrophil responses during the induction of arthritis in SKG mice. The neutrophilia we observed in SKG mice may be clinically relevant as we also observed a trend in the relationship between neutrophils and Bath Ankylosing Spondylitis Disease Activity Index score, which is a measurement of patient-specified pain and discomfort at any given moment (online supplemental figure 2A and table 1). Perhaps more relevant, is the relationship between neutrophils and the functional limitation (ie, disability) of AS patients as measured by the BASFI (Bath Ankylosing Spondylitis Functional Index). As shown in figure 4A, Patients with AS (selected from a larger cohort of SpA patients, online supplemental table 1) who were naïve to disease-modifying antirheumatic drugs (DMARDs) were longitudinally evaluated for percentage of neutrophils, absolute neutrophil count (ANC) and severity of disability, as determined by the BASFI. On average, for every increment of 5000 neutrophils/mm^3 the disability status worsened by ~15% (ΔBASFI of 1.5). Moreover, the positive correlation between neutrophils and disease severity was similarly observed in the complete SpA cohort (AS, psoriasis and psoriatic arthritis; online supplemental figure 2B,C). We further detected that ANC in DMARD-naïve SpA patients with early-stage disease (ie, diagnosed <5 years of first symptom) were statistically distinguished from patients in later stages of disease (>5 years past diagnosis) (online supplemental figure 2D). Collectively, our data support an intriguing role for neutrophils in the induction of Th17-mediated forms of SpA including AS.

Thus, to elucidate a causal mechanism for neutrophils in the onset of arthritis and induction of Th17 cells, we examined SKG mice deplete of neutrophils (figure 4B,C). Neutrophil-deficient SKG mice had impaired onset of arthritis (figure 4B) and a significant reduction in Th17 cells (figure 4D) like what we observed in Card9$^{-/-}$SKG mice. Cumulatively, these data reveal a novel neutrophil-specific function in the peripheral expansion of arthritogenic Th17 cells and onset of arthritis in SKG mice.

Card9-signalling within neutrophils promotes their activation and ability to expand arthritogenic Th17 cells

Thus far, our data identify a role for Card9 in controlling neutrophil responses, and that neutrophils in turn, control induction of Th17 cells and onset of arthritis in SKG mice. Consistent with an acute Card9/neutrophil-coupled mechanism for onset of arthritis, we observed a significant reduction in neutrophils (figure 5A), induction of Th17 cells (figure 5B) and onset of

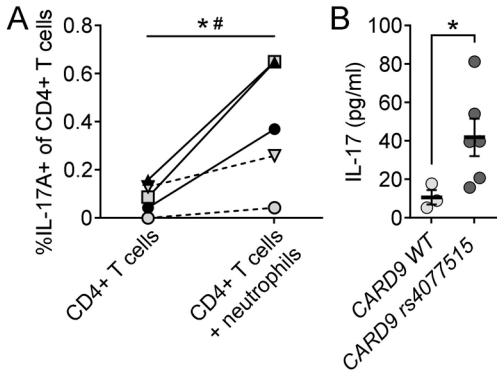

Figure 6 IL-17 production by AS patient T cells is potentiated by neutrophils and is positively associated with expression of CARD9 rs4077515. (A) Autologous cocultures with CD4$^+$ T cells and neutrophils derived from HLA-B27$^+$ AS patients were carried out for 72 hours, and the percentage of CD4$^+$ T cells producing IL-17A was determined by flow cytometry. Data were analysed by paired t test, $^{\#}p<0.05$, $^*p<0.05$, where # indicates the comparison between T cells only and T cells+neutrophils for all patients and * for only patients diagnosed <5 years. Solid lines indicate patients diagnosed <5 years and dotted >5 years. (B) Serum IL-17A was quantified by ELISA in biological DMARD naïve, HLA-B27+AS patients (n=9) expressing either the wild-type *CARD9* gene or the rs4077515 CARD9 variant. Mann-Whitney rank sum test, $^*p<0.05$. AS, ankylosing spondylitis.

arthritis (figure 5C) in Card9$^{-/-}$SKG mice versus SKG mice within the first 5 days post-zymosan.

To elucidate a direct cellular function for Card9 within neutrophils in controlling their activation status and ability to expand arthritogenic Th17 cells, we employed a reductionist approach *in vitro* with autologous neutrophil:CD4+T cell cocultures. Neutrophils purified from naïve SKG or Card9$^{-/-}$SKG mice were prestimulated with zymosan and then combined with CD4$^+$ T cells that were derived from naïve SKG mice. Responses to TCR-ligation by CD3/CD28 were then evaluated over the course of 72 hours (figure 5D–H). On TCR-ligation SKG T cells similarly expanded in the presence of both SKG and Card9$^{-/-}$SKG neutrophils, which was significantly greater compared with SKG T cells stimulated in the absence of neutrophils (ie, 'none'; figure 5D). These data underscore a role for neutrophils in directly potentiating proliferation of arthritogenic T cells. Viability remained the same for both SKG and Card9$^{-/-}$SKG neutrophils within the first 18 hours (figure 5E), but then as expected declined given the short life expectancy of activated neutrophils in culture. We did, however, note that zymosan-activated Card9$^{-/-}$SKG neutrophils had an advantage over SKG neutrophils in their survival; thereby revealing a role for Card9 within neutrophils in promoting cell death.

We further evaluated the cellular responses of Card9-deficient neutrophils in co-culture with T cells (figure 5F,G). Cell surface expression of Dectin-1 on neutrophils was similarly downregulated (figure 5F), indicating sensing of zymosan and Dectin-1 activation. However, Card9$^{-/-}$SKG neutrophils had impaired activation status as indicated by retention of CD62L, decreased CD63, MHCII and CD11b (figure 5G). These data demonstrate a direct cellular function of Card9-signalling within neutrophils in promoting their activation status and death. Lastly, we evaluated whether neutrophils directly control IL-17A production by SKG T cells, and if Card9-deficiency mediates this interaction (figure 5H). Consistent with the ability of neutrophils from SKG mice to expand SKG T cell proliferation (as shown in figure 5D), they also significantly increased IL-17 production within 18 hours of TCR-ligation that was extended out to 72 hours versus SKG T cells not cultured with neutrophils (ie, 'none' control). As a control, SKG or Card9$^{-/-}$SKG neutrophils cultured in the absence of SKG T cells under the same conditions failed to produce measurable amounts of IL-17A (online supplemental figure 3); thereby supporting the TCR-specificity of IL-17A production. Importantly, SKG T cells produced significantly reduced amounts of IL-17A production when cocultured with Card9$^{-/-}$SKG neutrophils. Collectively, these data reveal a novel, neutrophil-intrinsic function of Card9 in directly potentiating neutrophil activation and IL-17A production by SKG T cells as the cellular mechanism by which Card9 drives arthritis in SKG mice.

IL-17 production by AS patient T cells is potentiated by neutrophils and is controlled by proper CARD9 function

The clinical relevance of the Card9/neutrophil coupled mechanism and Th17 induction uncovered here in SKG mice, was further extended to patients with AS. Autologous cocultures with neutrophils and CD4$^+$ T cells derived from patients with AS (figure 6A; patient demographics reported in online supplemental table 2). We observed that neutrophils significantly potentiated IL-17A production in T cells (~6–13 fold compared with that of the response of CD4$^+$ T cells alone (figure 6A). In line with our *in vivo* and *in vitro* observation that support early, acute neutrophil responses in the induction of arthritis, we noted that neutrophils derived from AS patients with early onset disease (solid lines) produced greater amounts of IL-17A production compared with patients diagnosed >5 years (dashed lines). These data support the pathogenic role for peripheral neutrophil responses in induction of Th17 responses in AS patients, which would also be consistent with early onset SpA patients having higher neutrophil counts (online supplemental figure 2C). Importantly, biological DMARD-naïve, HLA-B27$^+$ AS patients with the CARD9 rs4077515 variant had significantly greater amounts of plasma IL-17A compared with HLA-B27$^+$ AS patients expressing the endogenous (WT) form of CARD9 (figure 6B); further underscoring the importance of dysregulated CARD9 in IL-17A production in AS patients.

In conclusion, this report reveals a Card9/Th17 pathway in the induction of arthritis, which is mediated by a Dectin-1/Card9-coupled signalling response in neutrophils that directly controls activation and IL-17A production in T cells. This model seems feasible in AS patients rs4077515 variant expression, where neutrophils could potentiate IL-17A production and drive a Th17-mediated form of AS.

DISCUSSION

Despite its connection with AS, we know very little about how dysregulation of CARD9 participates in Th17-mediated pathology. This study provides insight into a neutrophil-specific function of Card9 as an upstream determinant of pathogenic Th17 responses that drive disease in patients with AS and in experimental AS in SKG mice. How the observed connection between proper CARD9 controls immune homeostasis and mutated CARD9 (rs4075515 variant) enhances IL-17A/Th17-responses is interesting to consider. The CARD9 risk allele rs4075515 is thought to result in a gain-of-function of CARD9 based on its expression being increased in blood cells[40] and peripheral blood mononuclear cells (PBMCs).[41] Mechanistically, we demonstrated that HLA-B27$^+$ AS patients expressing the CARD9 rs4075515 variant had significantly increased IL-17A compared with HLA-B27$^+$ AS patients with the endogenous (non-mutated) form of *CARD9*. In this context, one can envision how hyperactivation

of the CARD9-signalling axis could provoke Th17-mediated disease. This would be corroborated by our mechanistic studies *in vivo* showing how endogenous Card9 expression drives Th17-immunity in SKG mice. These data support the premise for a Card9/Th17-coupled disease mechanism, and that patients with the CARD9 rs4075515 allele would respond more favourably to IL-17A-inhibitory biologics compared with AS patients with endogenous *CARD9*. Future studies would demonstrate the feasibility of using CARD9 rs4075515 as a predictive marker for patient responsiveness to IL-17A-inhibition.

Card9 is an important antifungal signalling hub. It was somewhat surprising, therefore, that disease in SKG mice was triggered in a Dectin-1 specific manner. Dectin-2, another essential CLR during fungal infection[42] activates the Card9-pathway[43]; yet did not trigger arthritis in SKG mice. This might shed light onto an especially unique Dectin-1/CARD9 coupled signalling mechanism in the way environmental fungi and *CARD9* genetic predisposition cause AS. CARD9 is also vital for control of Mtb infection,[28] and Mtb is the key constituent of CFA where Mincle activation has recently been shown to be responsible for the induction of Th17-mediated types experimental autoimmunity such as uveitis and multiple sclerosis.[29 30] It was therefore surprising that neither Mtb nor TDB were capable of triggering disease in SKG mice, indicating that activation of Card9 is not necessarily sufficient to trigger arthritis in SKG mice. In line with this, other CLRs, NLRs and TLRs, are known to play vital roles in host-defence against fungal species; yet only the loss of DECTIN-1 and CARD9 are associated with susceptibility against rampant fungal infection in people. Accordingly, patients with *CARD9* loss-of-function mutations succumb to systemic fungal disease, which is primarily caused by Dectin-1 triggering *Candida* species.[44] Thus, it is intriguing to consider that a *CARD9* variant which confers protection against fungal and mycobacteria species would be prioritised evolutionarily, but at the potential cost of risk in development of AS. Indeed, expression of the CARD9 variant rs4075515 is prevalent in Caucasians (53%) and Africans (25%)[45]; whereas loss-of-function *CARD9* mutations, which result in systemic *candidiasis*, are exceedingly rare.[46]

That we place Card9 within neutrophils as the upstream cellular mediator of induction of pathogenic Th17 responses adds further insight into our understanding of AS as a 'mixed pattern' condition,[9] where innate cellular responses shape a T cell-mediated disease. A break in central tolerance and generation of autoreactive T cells is the underlying mechanism of disease in SKG mice. However, Card9 was dispensable for T cell development and T cell function, which contrasts with the paradigm that has been built around HLA-B27. Clinically, neutrophils have been associated with other Th17-mediated autoimmune diseases including psoriatic arthritis[47] and rheumatoid arthritis[48]; thereby underscoring an important neutrophil/T cell connection in Th17-mediated diseases. Prior studies: although limited, support neutrophilia in AS patients, and our studies support a correlation between and disease severity. Further, we have found that neutrophils also correlated with spondyloarthropathy patients with psoriatic arthritis as well (online supplemental figure 3), supporting a generalised neutrophilia in both AS and SpAs. A major contribution for myeloid cells (including monocytes, macrophages and neutrophils) in arthritis has been proven experimentally in SKG mice.[35 36] Due to our usage of 1A8, which only depletes neutrophils and leaves monocytes intact, our data demonstrate a neutrophil-specific requirement in SKG mice. In line with an early contribution of neutrophils, neutrophil infiltration within axial and peripheral entheseal sites in SKG mice was reported within the first week of.[49] Thus, these data reveal a novel function of acute neutrophil responses within the periphery in early induction in Th17-responses and arthritis versus the conventional concept that Th17 cells recruit neutrophils, which subsequently exacerbate later-stages disease within the joints. Thus, it is entirely possible that neutrophils function in a bidirectional manner to both induce and/or exacerbate Th17 responses based on stage of disease.

While this work contributes to neutrophil/Th17-coupled response during AS, the novelty lies the neutrophil-intrinsic function of Card9 in directly controlling T cellular production of IL-17A in SKG mice and patients with AS. We are aware of limited data supporting neutrophil/Card9-coupled mechanism in rheumatic disease models. In support of our findings Card9 was recently reported in an immune complex model of dermatitis[50] and arthritis,[38] where a neutrophil-intrinsic function of Card9 was been observed. An important distinction, however, is that these disease models were not Th17-mediated, nor did the mice develop SpA/AS. Our data uncovered a potential role for Card9 in promoting neutrophil death in the cocultures, in that Card9-deficiency promoted neutrophil survival. This would support Card9 as a potential check-point inhibitor of neutrophils to mitigate potentiation of pathogenic Th17 responses and autoimmunity.

In conclusion, our collective observations shed light onto the spectrum of innate, 'autoinflammatory' and T cell, 'autoimmune' pathologies that can coexist in AS. We would posit an 'autoinflammatory' role for Card9 within neutrophils in mitigating hyper-responsiveness of autoreactive T cells that produce IL-17. This work exemplifies the of 'yin-yang' relationship of evolutionarily conserved host defence responses (ie, CARD9-mediated fungal protection) that when enhanced (ie, gain-of-function CARD9 rs4075515), may increase neutrophil activation and function important in clearing *C. albicans* infection, yet when dysregulated may contribute to loss of tolerance and autoimmunity.

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Patient consent for publication Not applicable.

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