

CARD9-dependent macrophage plasticity regulates effective fungal clearance

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J Clin Invest. 2025. <https://doi.org/10.1172/JCI188827>.

Research In-Press Preview Dermatology Immunology Infectious disease

The role of CARD9 in the pathogenesis of various chronic fungal infections has been established; however, the precise mechanisms underlying the pathobiology of these infections remain unclear. We aimed to investigate the specific cellular mechanisms by which CARD9 deficiency contributes to the pathogenesis of chronic fungal infections. Using single-cell RNA sequencing (scRNA-seq), we analyzed the immune cell profiles in skin lesions from both murine and human samples. We focused on macrophage differentiation and signaling pathways influenced by CARD9 deficiency. We found that CARD9 deficiency promotes the differentiation of TREM2^{high} macrophages following fungal stimulation, impairing their antifungal functions and inducing exhaustion-like T helper 1 (Th1) cells. Mechanistically, the NF- κ B pathway activation was restricted in CARD9-deficient macrophages, leading to enhanced CREB activation, which in turn exerted a positive regulatory effect on *Trem2* expression by activating C/EBP β . Notably, targeting TREM2 enhanced the antifungal immune response *in vivo* and *in vitro*, thereby alleviating the severity of CARD9-deficient subcutaneous dematiaceous fungal infection. Our findings highlight the important role of CARD9 in regulating cutaneous antifungal immunity and identify potential targets for immunotherapy in chronic dematiaceous fungal infections.

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1 **CARD9-dependent macrophage plasticity regulates effective fungal clearance**

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9 **Conflict of interest:** The authors have declared that no conflict of interest exists.

10

1 **Abstract**

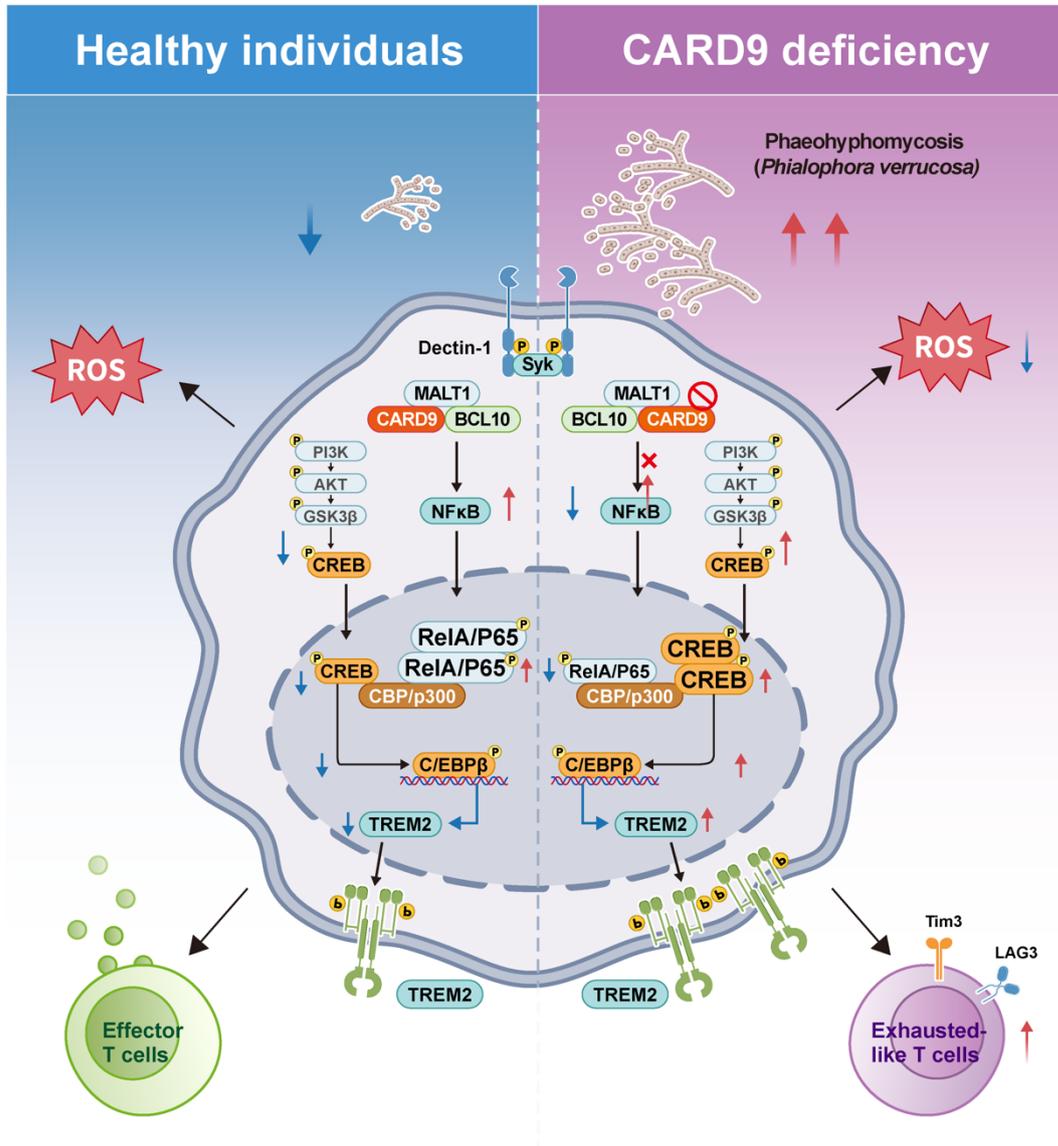
2 The role of CARD9 in the pathogenesis of various chronic fungal infections has been established;
3 however, the precise mechanisms underlying the pathobiology of these infections remain unclear.

4 We aimed to investigate the specific cellular mechanisms by which CARD9 deficiency contributes
5 to the pathogenesis of chronic fungal infections. Using single-cell RNA sequencing (scRNA-seq),
6 we analyzed the immune cell profiles in skin lesions from both murine and human samples. We
7 focused on macrophage differentiation and signaling pathways influenced by CARD9 deficiency.

8 We found that CARD9 deficiency promotes the differentiation of TREM2^{high} monocyte-derived
9 macrophages following fungal stimulation, impairing their antifungal functions and inducing
10 exhaustion-like T helper 1 (Th1) cells. Mechanistically, the NF-κB pathway activation was
11 restricted in CARD9-deficient macrophages, leading to enhanced CREB activation, which in turn
12 exerted a positive regulatory effect on *Trem2* expression by activating C/EBPβ. Notably, targeting
13 TREM2 enhanced the antifungal immune response *in vivo* and *in vitro*, thereby alleviating the
14 severity of CARD9-deficient subcutaneous dematiaceous fungal infection. Our findings highlight
15 the important role of CARD9 in regulating cutaneous antifungal immunity and identify potential
16 targets for immunotherapy in chronic dematiaceous fungal infections.

17

1 Graphical abstract



2

1 **Introduction**

2 Fungal infections represent a crucial and growing global health concern owing to various pathogens
3 and clinical manifestations (1-3). Recent estimates indicate that over 6.55 million individuals
4 worldwide face life-threatening fungal infections annually (4) underscoring the magnitude of this
5 issue. With advances in genetics and immunology, the critical role of genetic susceptibility in
6 patients has been uncovered, particularly those with deficiencies in caspase recruitment domain-
7 containing protein 9 (CARD9), which are associated with severe intractable fungal infections and
8 high mortality rates (1, 5, 6). CARD9 is a crucial signaling adaptor that functions downstream of
9 several C-type lectin receptors (CLRs) and plays a vital role in host immune responses against
10 fungal pathogens (5, 7, 8). However, the comprehensive mechanisms by which CARD9 influences
11 antifungal immunity have not been fully elucidated.

12 Macrophages, the primary cell type expressing CARD9, play a crucial role in antifungal
13 immunity via direct and indirect mechanisms (9-11). Previous studies have shown that while loss of
14 CARD9 markedly impairs the fungicidal capacity of macrophages (12-16), it does not notably affect
15 their recruitment or phagocytic functions (17). However, more recent research using the candidiasis
16 model reported that CARD9 deficiency led to defective monocyte aggregation at day 1 post-
17 infection, followed by abnormal accumulation of Ly6C⁺ monocytes and MHCII⁺Ly6C⁺ monocyte-
18 derived cells by day 4 in the infected brain (18). These findings highlight inconsistencies in the
19 reported effects of CARD9 deficiency on macrophage-mediated antifungal responses across
20 different infection models. Triggering receptor expressed on myeloid cells-2 (TREM2), a myeloid
21 cell surface receptor, has been identified as an important immune signaling hub in several
22 pathological conditions (19, 20). Its effect on macrophage function remains a topic of considerable

1 debate, with evidence suggesting that TREM2 exerts opposing effects in different disease states.
2 Some studies have suggested that TREM2 negatively regulates Toll-like receptor (TLR) signaling,
3 thereby suppressing proinflammatory mediator secretions and anti-infective functions (20-25).
4 Conversely, recent studies have indicated that TREM2 can induce bacterial phagocytosis, which is
5 crucial for pathogen clearance and inflammation onset (26, 27). However, the influence of TREM2
6 on host antifungal immune function remains unexplored and warrants further investigation.

7 Host immune responses to fungal pathogens involve a complex interplay between innate and
8 adaptive immunity. Adaptive immunity, particularly T-helper 1 (Th1)- and Th17-related cellular
9 responses, is crucial for robust antifungal capabilities (28-30). CARD9 serves as a critical bridge
10 between the innate and adaptive immunity. Previous research has suggested that CARD9 deficiency
11 impairs Th1 and Th17 cell differentiation and compromises essential cytokine secretions such as
12 IFN- γ , IL-17A, and IL-22, thereby weakening the adaptive antifungal immune response in the host
13 (6, 15, 31, 32). Nevertheless, some CARD9-deficient patients exhibit normal Th17 cell
14 differentiation (33-38). These inconsistencies highlight the need for further investigations into how
15 CARD9 deficiency affects adaptive antifungal immunity.

16 Given the high prevalence and treatment resistance of dematiaceous fungal infections in
17 patients with CARD9 deficiency at our center, we employed single-cell RNA sequencing (scRNA-
18 seq) to investigate the local immune landscape in the skin lesions of both murine models and human
19 patients with this infection. This study revealed the function of CARD9 in regulating the
20 differentiation of macrophages by modulating the balance between the NF- κ B/P65 and CREB-
21 C/EBP β pathways. Consequently, TREM2^{high} macrophages are enriched in CARD9-deficient
22 individuals, which impacts innate and adaptive antifungal immune responses. Moreover, the

1 administration of TREM2 agonists can delay the progression of CARD9-deficient dematiaceous
2 fungal infections. In conclusion, our findings reveal the regulatory mechanisms underlying TREM2
3 expression and its influence on antifungal immune responses, identifying a potential target for
4 immunotherapy in patients with chronic CARD9-related dematiaceous fungal infections.

1 **Results**

2 **CARD9 is necessary for defense against subcutaneous dematiaceous fungal infection**

3 Previous studies indicated that CARD9-deficient patients are susceptible to severe dematiaceous
4 fungal infections; however, its underlying mechanisms remain poorly understood. *Phialophora*
5 *verrucosa* is the most commonly identified causative fungus in CARD9-deficient patients with
6 phaeohyphomycosis (32). To investigate the role of CARD9 in shaping host protective immunity
7 against dematiaceous fungi, we first modeled subcutaneous phaeohyphomycosis with footpad
8 inoculation of *P. verrucosa* in wild-type (WT) and *Card9*-knockout (*Card9*^{-/-}) mice. Following
9 fungal inoculation, the two groups exhibited different patterns of footpad swelling. WT mice showed
10 more pronounced swelling on day 3, with comparable levels between groups on day 7, followed by
11 gradual resolution. However, *Card9*^{-/-} mice experienced progressive deterioration, with increased
12 swelling on day 10 and more pronounced swelling on day 14 and thereafter (Figure 1, A and B). To
13 elucidate the cellular and molecular mechanisms underlying CARD9-mediated antifungal immune
14 responses during subcutaneous *P. verrucosa* infection, we conducted scRNA-seq of total cells in
15 mouse footpads at 3, 7, 10, and 14 d post-infection (Figure 1B). Following data preprocessing and
16 quality control, we partitioned the cells into 15 major clusters and labeled them based on
17 representative marker genes, including ten immune cell clusters and five non-immune cells (Figure
18 1C). Overall, on day 3 post-infection, neutrophils and monocytes/macrophages were the
19 predominant immune cells in both mouse strains, with *Card9*^{-/-} mice showing fewer neutrophils and
20 more monocytes/macrophages than WT controls (Figure 1D). On day 7 post-infection, the
21 proportions of T and NK cells increased in the skin lesions of WT mice and remained elevated
22 through days 10 and 14 (Figure 1D). In contrast, *Card9*^{-/-} mice showed reduced T and NK cell

1 infiltration but an increased proportion of eosinophils in the lesions compared to WT mice (Figure
2 1D). To elucidate the role of CARD9 in modulating the recruitment and function of local immune
3 cells in lesions, we analyzed the distribution of *Card9*-expressing cells in this model. Macrophages
4 constituted the predominant population, comprising 61.69% of *Card9*-positive cells (Figure 1E).
5 This predominant expression underscores macrophages as the pivotal cellular subset for subsequent
6 in-depth analysis.

7

8 **TREM2^{high} macrophages display anti-inflammatory signatures and are increased in *Card9*^{-/-}**
9 **mice**

10 Macrophages, a pivotal cell type in antifungal immunity, are among the main expressers of CARD9.
11 We conducted further subpopulation analyses and identified five distinct macrophage subsets:
12 *Cxcl3*^{high} macrophage, *Ccl5*^{high} macrophage, *Trem2*^{high} macrophage, *Il10*^{high} macrophage, and
13 *Mrc1*^{high} macrophage (Figure 2A). The proportions of *Cxcl3*^{high} macrophage and *Ccl5*^{high}
14 macrophage were higher in WT mice, whereas the TREM2^{high} macrophage subset was considerably
15 more abundant in *Card9*^{-/-} murine lesions (Figure 2B). Notably, the TREM2^{high} macrophage subset
16 was characterized by high expression of *Trem2*, *Lgals3*, and *Spp1* (Figure 2C), a transcriptional
17 profile consistent with the gene signature previously described for skin monocyte-derived
18 macrophages (39). In addition, the analysis of monocyte-associated gene programs across
19 macrophage subsets provided further indications that this population represents a monocyte-derived
20 macrophage lineage (Supplementary Figure 1A). HALLMARK gene set scoring among the major
21 macrophage subsets revealed that the TREM2^{high} macrophages exhibited notably reduced activities
22 in the NF-κB signaling pathway and pathways related to pro-inflammatory cytokines, such as TNF-

1 α , IFN- γ , and IL-6 (Figure 2D). Further flow cytometry analysis demonstrated that the proportion
2 of TREM2⁺ macrophages among all macrophages was markedly higher in *Card9*^{-/-} murine lesions
3 than in WT murine lesions on day 10 post-infection (Figures 2, E and F), whereas no differences
4 were observed prior to infection (Supplementary Figure 1B). Multiplex immunofluorescence
5 (mIHC) experiments also showed higher TREM2 expression and its co-localization with the
6 macrophage marker F4/80 in lesions of *Card9*^{-/-} mice (Figure 2, G and H). The collective findings
7 indicate that CARD9 deficiency does not notably affect macrophage recruitment to the infection
8 site but rather substantially alters macrophage phenotypes in response to subcutaneous *P. verrucosa*
9 infection. A similar pattern of increased recruitment but impaired functional responses has been
10 reported in CARD9-deficient mice challenged with *C. albicans* (18). Together, these observations
11 suggest that CARD9 regulates macrophage plasticity, potentially impairing antifungal effector
12 functions.

13 Neutrophils are another type of immune cell that mainly expresses CARD9 and plays a critical
14 role in antifungal immunity. We performed a detailed subpopulation analysis of neutrophils to
15 investigate their heterogeneity, identifying eight subsets (Supplemental Figure 1C). Neu-C1,
16 characterized by high expression of *S100a9* and *Nfkb1*, and Neu-C2, enriched for *Tnf* and *Il23a*,
17 were predominantly enriched in the WT mice, whereas Neu-C4, marked by elevated *ApoE* and *Il10*,
18 was more abundant in the *Card9*^{-/-} mice. (Supplemental Figure 1, D-F). Subsequent comparative
19 analyses further revealed that multiple pro-inflammatory cytokines and chemokines including *Il1b*,
20 *Tnf*, *Ccl5*, *Cxcl9*, and *Cxcl10*, were downregulated in *Card9*^{-/-} group (Supplemental Figure 1G).
21 Gene ontology (GO) enrichment analysis consistently indicated that key pathways associated with
22 cytokine production, reactive oxygen species (ROS) response, and other immune-related processes

1 were suppressed in *Card9*^{-/-} neutrophils (Supplemental Figure 1H). Collectively, these findings are
2 consistent with those of previous studies, supporting the notion that CARD9 is essential for
3 maintaining neutrophil function in antifungal immunity, and future studies may help to elucidate its
4 detailed regulatory mechanisms.

5

6 **Exhaustion-like Th1 cells are more pronounced in *Card9*^{-/-} mice and correlate with anti-**
7 **inflammatory macrophages**

8 CARD9 has been shown to regulate the activation of innate immune cells and the production
9 of cytokines, thereby shaping adaptive T cell responses. Due to the altered macrophage phenotypes
10 and reduction in T cells observed in *Card9*^{-/-} mice infected with *P. verrucosa*, we conducted a
11 subpopulation analysis of T cells (Figure 3A). We found that *Card9*^{-/-} mice exhibited a higher
12 frequency of regulatory T cells (Tregs) and Th2 cells but displayed a lower proportion of Th1 cells
13 than WT mice (Figure 3B, Supplemental Figure 2A). To gain further insight into the functional
14 status of the predominant T cell subsets, we performed a pseudo-time trajectory analysis of Th1
15 cells (Figure 3C). The results revealed a progressive shift from a naïve state through an effector
16 phenotype and ultimately to an exhausted state during infection (Figure 3, C and D). Notably, *Card9*
17 ^{-/-} mice displayed a greater accumulation of exhaustion-like Th1 cells within the lesions on day 10
18 and 14 post-infection (Figure 3E and Supplemental Figure 2, B and C). Flow cytometry analysis
19 confirmed that CD4⁺ T cells in *Card9*^{-/-} murine lesions exhibited markedly higher expression of
20 immune checkpoints than those in WT controls on day 10 post-infection (Figure 3, F–H), whereas
21 no differences were observed prior to infection (Supplemental Figure 2D). Furthermore, mIHC
22 revealed a greater degree of co-localization between these immune checkpoints and the CD4⁺ T cell

1 population in lesions of *Card9*^{-/-} mice on day 10 post-infection (Supplemental Figure 2, E and F).
2 Collectively, these results suggest that CARD9 plays a critical role in regulating T cell recruitment
3 and function during dematiaceous fungal infections. Specifically, CARD9 deficiency promotes the
4 accumulation of immunosuppressive Treg cells and exhaustion-like Th1 cells.

5 Given that CARD9 is predominantly expressed in myeloid rather than lymphoid cells, the
6 observed alteration in T cells is likely to be a secondary effect of altered myeloid cell function. The
7 interactome analysis of primary immune cell populations revealed that macrophages exhibited the
8 most obvious interactions with T cells (Supplemental Figure 2G). Previous studies have shown that
9 certain macrophage subsets can promote T cell exhaustion (40, 41). We further examined the
10 interactions between major macrophage subpopulations and exhaustion-like Th1 cells; the results
11 indicated that TREM2^{high} macrophages displayed higher interactions with exhaustion-like Th1 cells
12 (Figure 3I). Additionally, we analyzed the ligand–receptor interactions between macrophage
13 subpopulations and exhausted Th1 cells. Macrophages from the *Card9*^{-/-} group demonstrated
14 stronger interactions with exhausted Th1 cells compared to those from the WT group. In particular,
15 ligand–receptor pairs such as *Tgfb1*-(*Tgfbr1*+*Tgfbr2*), *Lgals3*-*Lag3*, and *Cd274*-*Pdcd1* exhibited a
16 marked increase in signaling strength, suggesting that these enhanced interactions may contribute
17 to the promotion of Th1 cell exhaustion in the *Card9*-deficient condition (Figure 3J). These findings
18 further support the notion that CARD9 deficiency promotes the accumulation of anti-inflammatory
19 macrophages, which may contribute to the impaired T-cell responses observed in lesions of *Card9*^{-/-}
20 mice.

21

22 **TREM2^{high} macrophages in lesions of CARD9-deficient patient with dematiaceous fungal**

1 **infection**

2 Building on the murine infection model findings, we validated our observations in a CARD9-
3 deficient patient with a subcutaneous dematiaceous fungal infection. Three distinct macrophage
4 subpopulations were identified (Figure 4A). The most abundant subset was TREM2^{high}
5 macrophages, whose transcriptional profile closely resembled those in murine lesions (Figure 4, A
6 and B). In contrast, analysis of skin tissues from three healthy controls revealed minimal TREM2
7 expression in macrophages (Figure 4C). Further, mIHC analysis confirmed the high TREM2
8 expression and its co-localization with the macrophage marker CD68 in skin lesions from three
9 CARD9-deficient patients with dematiaceous fungal infections, compared to lesions from three
10 healthy controls (Figure 4, D and E).

11 T cell analysis showed that Tregs were the largest CD4⁺ T cell subset (Supplemental Figure 3,
12 A and B), with a notable proportion of the remaining CD4⁺ and CD8⁺ T cells exhibiting signs of
13 exhaustion, characterized by a high immune checkpoint expression and elevated exhaustion scores
14 (Figure 4, F and G). Using mIHC, we further confirmed that the immune checkpoints TIM3 and
15 LAG3 were notably colocalized with CD4 in the skin lesions of three CARD9-deficient patients
16 with subcutaneous dematiaceous fungal infection, in comparison with those of three healthy controls
17 (Supplemental Figure 3C). Moreover, we analyzed the receptor-ligand interactions between
18 macrophage subsets and exhaustion-like CD4⁺ T cells. This analysis corroborated our findings in
19 the mouse model, demonstrating that the TREM2^{high} macrophage subset exhibited the most
20 prominent interactions with exhaustion-like CD4⁺ cells (Figure 4H). In conclusion, these findings
21 strongly validate our findings in a mouse infection model, indicating that CARD9 deficiency leads
22 to the differentiation of anti-inflammatory macrophages, which may contribute to impaired T cell

1 responses in human skin lesions of dematiaceous fungal infection.

2

3 **CARD9 deficiency induces high TREM2 expression in macrophages and impairs antifungal**
4 **infection**

5 To further determine the mechanisms underlying the accumulation of TREM2^{high} macrophages in
6 CARD9-deficient skin lesions, we conducted RNA sequencing to analyze the transcriptional profile
7 of bone marrow-derived macrophages (BMDMs) derived from WT and *Card9*^{-/-} mice stimulated
8 with heat-killed *P. verrucosa* for 24 h. Several genes exhibited differential expression patterns
9 between WT and *Card9*^{-/-} BMDMs (Figure 5A). The upregulated genes in *Card9*^{-/-} BMDMs
10 included *Trem2*, *Lgals3*, and *ApoE*, which are characteristics of the TREM2^{high} macrophage subset
11 identified in the *in vivo* infection model (Figure 5A and Figure 2D). Additionally, the expression of
12 anti-inflammatory cytokine, including *Tgfb1*, *Tgfb3*, and *Il10*, was also higher in *Card9*^{-/-} BMDMs
13 (Figure 5A). In contrast, the upregulated genes in WT BMDMs included *Ccl5* and *Cxcl3*, which
14 were specifically expressed in *Cxcl3*^{high} macrophage and *Ccl5*^{high} macrophage subsets observed *in*
15 *vivo* (Figure 5A and Figure 2D). Furthermore, the expression of pro-inflammatory cytokines and
16 chemokines, such as *Il1b*, *Tnf*, *Cxcl1*, and *Cxcl2*, was higher in WT BMDMs (Figure 5A). Western
17 blotting was performed to validate the differential expression of TREM2 in BMDMs. *Card9*^{-/-}
18 BMDMs exhibited substantially higher TREM2 levels than WT BMDMs after heat-killed *P.*
19 *verrucosa* simulation (Figure 5, B and C). Additionally, ELISA analysis of culture supernatants
20 revealed no difference in the levels of soluble TREM2, suggesting that the increased expression
21 observed by western blot primarily reflects the membrane-bound form, rather than enhanced
22 secretion (Supplemental Figure 4A). Similarly, the knockdown of endogenous CARD9 in THP-1

1 cells resulted in consistent differential expression patterns. RNA sequencing and immunoblotting
2 confirmed *P. verrucosa*-induced higher expression of TREM2 in CARD9-knockdown THP-1 cells
3 (Figure 5, D–F).

4 Subsequently, we conducted a KEGG enrichment analysis to compare the activation of *P.*
5 *verrucosa*-induced signaling molecules in WT and *Card9*^{-/-} BMDMs. These results demonstrated
6 markedly diminished activation of NF- κ B signaling in *Card9*^{-/-} BMDMs (Supplemental Figure 4B).
7 By contrast, activation of the PI3K/AKT signaling pathway was greater in *Card9*^{-/-} BMDMs than in
8 WT BMDMs (Supplemental Figure 4B), suggesting that CARD9 played a pivotal role in regulating
9 the equilibrium between NF- κ B and PI3K/AKT signaling pathways. Previous studies have revealed
10 that TLRs trigger anti-inflammatory signaling via the PI3K/AKT/GSK3 β pathways in macrophages,
11 which converge to activate CREB (42, 43). The relative amounts of active nuclear CREB and NF-
12 κ B p65 determine subsequent association with the nuclear coactivator CBP/p300, thereby regulating
13 the pro-inflammatory and anti-inflammatory responses in macrophages (43, 44). To elucidate
14 whether the anti-inflammatory phenotype of *Card9*^{-/-} BMDMs is associated with alterations in NF-
15 κ B and PI3K/AKT signaling, we conducted immunoblotting assays to measure the activation of key
16 molecules in these pathways. Upon *P. verrucosa* stimulation, *Card9*^{-/-} BMDMs demonstrated lower
17 phosphorylation of the NF- κ B p65 subunit while exhibiting higher phosphorylation of Akt, GSK3 β ,
18 and CREB in comparison with WT BMDMs (Figure 5, G and H). Furthermore, pretreatment of WT
19 BMDMs with NF- κ B inhibitor followed by *P. verrucosa* stimulation resulted in a notable increase
20 in the phosphorylation of CREB (Figure 5, I and J, and Supplemental Figure 4C), as well as the
21 expression of TREM2 (Figure 5, K and L). These findings provide further evidence that, in
22 conditions of impaired NF- κ B signaling, CREB is activated and contributes to the transcriptional

1 upregulation of TREM2.

2 To further elucidate the role of TREM2 upregulation in CARD9-deficient macrophage, we
3 performed transcriptome sequencing of WT and *Card9*^{-/-} BMDMs following TREM2 knockdown
4 and 24-h *P. verrucosa* stimulation. Notably, a subset of genes downregulated in *Card9*^{-/-} BMDMs
5 exhibited increased expression upon TREM2 knockdown compared to WT BMDMs (Figure 5M).
6 These included pro-inflammatory factors, such as *Il1a*, *Cxcl5*, and *Cxcl9*, as well as the
7 antimicrobial peptide *Sl100A8* (Figure 5N). GO functional enrichment analysis revealed that the
8 upregulated genes in the si-TREM2 group were enriched in biological processes, including defense
9 response, response to stimulus, and inflammatory response (Figure 5O). Furthermore, KEGG
10 pathway enrichment analysis indicated marked enrichment of upregulated genes in cytokine-
11 cytokine receptor interactions and signaling pathways related to IL-17, TNF, and chemokines
12 (Supplemental Figure 4D). To more directly assess the functional role of TREM2^{high} macrophages
13 in anti-fungal immunity, we overexpressed TREM2 in RAW264.7 cells (Supplemental Figure 4, E
14 and F). In comparison with control cells, TREM2-overexpressing macrophages demonstrated
15 impaired fungicidal activity (Figure 5P) and decreased production of total ROS (Figure 5Q).

16 Single-cell interaction analysis suggests that TREM2^{high} macrophages may contribute to T cell
17 exhaustion under *Card9*-deficient conditions (Figure 3, I–K). Notably, *Card9*^{-/-} BMDMs exhibited
18 elevated expression of *Il10* and *Tgfb1*, two immunoregulatory cytokines that have been previously
19 implicated in driving T cell exhaustion(41, 45, 46). Consistent with the transcriptional data, western
20 blot analysis confirmed increased protein levels of IL-10 and TGF- β in *Card9*^{-/-} BMDMs under *P.*
21 *verrucosa* stimulation (Figure 5, R and S), providing further support for a *Card9*^{-/-} macrophage-
22 mediated mechanism promoting T cell exhaustion.

1 Collectively, these data indicate that in CARD9-deficient macrophages, *P. verrucosa*-induced
2 NF- κ B signaling activation is constrained, leading to enhanced activation of CREB and the
3 predominance of Trem2^{high} macrophages. Moreover, upregulation of TREM2 expression in
4 CARD9-deficient hosts is associated with impaired innate and adaptive antifungal function.

5

6 **CARD9 negatively regulates *Trem2* expression by activating C/EBP β**

7 To further elucidate the mechanism by which the CARD9-related pathway regulates the expression
8 of TREM2, we screened transcription factors (TFs) that can directly bind to the *Trem2* promoter in
9 both mouse and human cells using bioinformatic analysis; this ultimately led to the identification of
10 C/EBP β as the most promising candidate (Figure 6A). Activated CREB promotes the expression
11 and activation of C/EBP β . Western blot analysis was performed to confirm the increased activation
12 of C/EBP β in *Card9*^{-/-} BMDMs upon *P. verrucosa* stimulation in comparison with WT BMDMs
13 (Figure 6, B and C). Previous studies have also demonstrated that the activation of C/EBP β can
14 promote the anti-inflammatory polarization of macrophages (44, 47). To ascertain whether C/EBP β
15 can regulate *P. verrucosa*-induced expression of TREM2 in macrophages, siRNA was employed to
16 knockdown C/EBP β in BMDMs, which were then stimulated with *P. verrucosa*. Knockdown of
17 C/EBP β notably suppressed *P. verrucosa*-induced expression of TREM2 in *Card9*^{-/-} BMDMs
18 (Figure 6, D and E). To determine whether C/EBP β directly dictates the *Trem2* transcription, we
19 further utilized a dual-luciferase reporter assay, which revealed that C/EBP β overexpression in 293T
20 human embryonic cells resulted in *Trem2* promoter activation (Figure 6F). Similarly, we conducted
21 chromatin immunoprecipitation assays and observed C/EBP β binding to the promoter region of
22 *Trem2* in CARD9-deficient macrophages (Figure 6G). These results collectively indicate that

1 augmented C/EBP β signaling in CARD9-deficient macrophages can directly bind to the promoter
2 region of *Trem2*, thereby exerting a positive regulatory effect on its expression.

3

4 **Anti-TREM2 antibody improves the antifungal immune response *in vivo* and *in vitro***

5 To investigate the potential therapeutic benefits of targeting TREM2 in the treatment of
6 phaeohyphomycosis, WT and *Card9*^{-/-} mice were infected with *P. verrucosa* and treated with a
7 blocking antibody of TREM2 (Figure 7A). The administration of anti-TREM2 antibody delayed
8 disease progression and reduced the footpad swelling rate in *Card9*^{-/-} mice compared to that in the
9 control group (Figure 7B). However, no differences in footpad lesion phenotypes were observed
10 between the antibody treatment and control groups in WT mice (Figure 7B). Histological
11 examination of the footpad on day 21 post-infection showed a marked reduction in inflammatory
12 cell infiltration, smaller infectious granulomas, and a lower fungal burden (spores and hyphae) in
13 the lesions of *Card9*^{-/-} mice treated with anti-TREM2 antibody compared with that in non-treated
14 *Card9*^{-/-} mice (Figure 7C). Furthermore, treatment with an anti-TREM2 antibody resulted in a
15 notable reduction in local fungal loads in *Card9*^{-/-} mice on day 21 post-infection (Figure 7D).

16 Flow cytometry was performed to elucidate the cellular mechanisms underlying anti-TREM2
17 antibody treatment. In WT mice, no differences were observed in the proportions of different
18 macrophage subsets between the antibody-treated and control groups. However, in *Card9*^{-/-} mice,
19 the anti-TREM2 antibody treatment group showed a substantially lower proportion of TREM2⁺
20 anti-inflammatory macrophages than the control group (Figure 7, E and F). Pretreatment of TREM2-
21 overexpressing RAW 264.7 cell with the blocking antibody did not affect subsequent detection by
22 flow cytometry, confirming no epitope interference (Supplemental Figure 4E). Furthermore, the

1 anti-TREM2 antibody-treated group displayed reduced expression of immune checkpoints (TIM3
2 and LAG3) on CD4⁺ T cells compared to the non-treated group. In contrast, both treatment and
3 control groups of WT mice exhibited minimal expression of immune checkpoints on CD4⁺ T cells
4 within the lesions (Figure 7, G and H). These results indicate that anti-TREM2 antibody may serve
5 as a potential therapeutic strategy for enhancing host innate and adaptive immunity against fungal
6 infections in *Card9*^{-/-} mice.

7 Previous studies have demonstrated that CARD9-deficient macrophages exhibit defects in
8 killing *P. verrucosa* (48). To ascertain whether targeting TREM2 affects the fungal killing ability of
9 macrophages *in vitro*, siRNA was used to knock down the expression of TREM2. The knockdown
10 of endogenous TREM2 restored the spore-killing ability of *Card9*^{-/-} BMDMs, whereas it had no
11 effect on WT BMDMs (Figure 7I). Moreover, the generation of ROS is a pivotal effector mechanism
12 of macrophages in antifungal immunity. HALLMARK gene set scoring among the major
13 macrophage subsets indicated that the TREM2^{high} macrophage subsets exhibited a reduction in ROS
14 pathway function (Figure 2E), and over-expression of TREM2 in RAW 264.7 cell also showed
15 impaired ROS production (Figure 5Q). Consequently, we investigated the impact of targeting
16 TREM2 on total ROS production in macrophages. *Card9*^{-/-} BMDMs exhibited a markedly
17 diminished capacity to generate ROS upon stimulation with *P. verrucosa* compared with WT
18 BMDMs (Figure 7J). Importantly, the knockdown of TREM2 considerably enhanced the ROS
19 generation by *Card9*^{-/-} BMDMs (Figure 7K). These results provide further evidence for the crucial
20 role of the TREM2-mediated anti-inflammatory pathway in compromising antifungal immunity in
21 the context of CARD9 deficiency. Modulation of TREM2 signaling represents a promising strategy
22 to enhance the fungicidal ROS response and fungal killing by macrophages and improve host

1 defense against dematiaceous fungal infections in CARD9-deficient settings.

1 **Discussion**

2 Fungal infections have been increasing globally, presenting a considerable disease burden and
3 endangering public health. Over the past decades, our understanding of the mechanisms underlying
4 the host antifungal immune response deepened but remains inadequately delineated (1). To elucidate
5 how the immune system responds to fungal skin infections, we established a subcutaneous *P.*
6 *verrucosa* infection model. Using high-throughput scRNA-seq, we generated a detailed global
7 portrait of the local immune cell populations in infectious skin lesions and conducted preliminary
8 validation using lesions obtained from patients. This single-cell atlas of the antifungal immune
9 response establishes a crucial foundation for future investigations into the immunological
10 mechanisms underlying fungal diseases, advancing both our fundamental understanding and the
11 potential for targeted immunotherapeutic strategies.

12 As a key adaptor protein downstream of fungal pattern recognition receptors, CARD9
13 efficiently integrates recognition signals from multiple receptors and regulates host antifungal
14 immunity (7). CARD9 deficiency notably increases susceptibility to various fungal infections (49-
15 52). To further evaluate the role of CARD9 in host antifungal immunity, we conducted a
16 comparative analysis of immune cells in skin lesions of WT and *Card9*^{-/-} mice. The overall number
17 of local macrophages did not show a marked difference between the groups, suggesting that CARD9
18 does not affect macrophage recruitment in *P. verrucosa* subcutaneous infection. However, we
19 observed a notable alteration in macrophage phenotype. In both mice and patients with CARD9
20 deficiency, skin lesions exhibited a pronounced increase in the anti-inflammatory TREM2^{high}
21 macrophage subset, whereas pro-inflammatory macrophages were markedly diminished. TREM2
22 is a crucial receptor expressed on the surfaces of macrophages and other myeloid cells (19). It

1 mediates diverse downstream signaling pathways upon binding to various ligands, including lipids,
2 β -amyloid peptides, TDP-43, APOE, and galectin-3, among others. Previous studies have
3 demonstrated that TREM2 signaling promotes an anti-inflammatory, tissue-repairing phenotype in
4 macrophages, which can be detrimental to antimicrobial immunity (21, 22, 24, 25). TREM2 also
5 suppresses the release of inflammatory mediators by negatively regulating TLR signaling during
6 bacterial infections (53). However, the role of TREM2 in fungal infections has not been well-studied.
7 Our findings emphasize the crucial role of CARD9 signaling in regulating the equilibrium between
8 the pro- and anti-inflammatory macrophage phenotypes. Previous research has also proposed that
9 CARD9 mediates the induction of a pro-inflammatory M1 phenotype by β -glucan, and loss of
10 CARD9 promotes an anti-inflammatory M2 macrophage polarization, impairing antifungal
11 functions (17, 54). A recent study demonstrated that monocytic responses act as key protective
12 effectors in chronic central nervous system candidiasis, showing that CARD9 deficiency impairs
13 the early upregulation of activation markers on mononuclear phagocytes (18). In this study, we
14 demonstrated that Card9 deficiency markedly alters the phenotype and function of monocyte-
15 derived macrophages in a subcutaneous dermaticeous fungal infection model. Collectively, these
16 findings underscore the critical importance of CARD9 in shaping monocyte and monocyte-derived
17 cell plasticity and function across diverse fungal infection models.

18 Nonetheless, the present study has limitations. Lineage-tracing and fate-mapping strategies
19 were not employed to directly determine the developmental origin of macrophage subsets, and thus
20 definitive evidence cannot be provided that the TREM2^{high} population is monocyte-derived.
21 However, by integrating previously reported gene signatures of skin monocyte-derived
22 macrophages, it was found that the transcriptional profile of the TREM2^{high} subset closely aligns

1 with this lineage (39). Furthermore, our *in vitro* experiments using BMDMs, RAW264.7 cells, and
2 THP-1–derived macrophages consistently demonstrated that CARD9 regulates TREM2 expression
3 and functional programs in monocyte-derived macrophages.

4 CARD9 is a critical mediator that links innate and adaptive immunity, and CARD9 deficiency
5 impairs T-cell differentiation. In this study, we observed a notable reduction in local T cell
6 infiltration within the lesions of *Card9*^{-/-} mice compared to those in WT controls. Additionally, there
7 was an increased frequency of regulatory Tregs and Th2 cells and a lower proportion of Th1 cells,
8 indicating that CARD9 plays a crucial role in mobilizing adaptive T-cell responses against fungal
9 infection. Furthermore, we revealed the development of an exhaustion-like phenotype in both CD4⁺
10 and CD8⁺ T cells within fungal infection lesions, characterized by elevated inhibitory receptor
11 expression, which was more pronounced in *Card9*^{-/-} mice. T-cell exhaustion is typically associated
12 with inadequate control and progression of chronic infections (55, 56). However, its role in fungal
13 infections remains poorly understood, with only a few studies demonstrating increased expression
14 of immune inhibitory receptors in systemic fungal infections (57-59). This study provides the
15 evidence of exhaustion-like changes in T cells during cutaneous dematiaceous fungal infections.
16 Analysis of cellular interactions indicated that the interplay between anti-inflammatory
17 macrophages and exhaustion-like Th1 cells may be one of the reasons for the induction of
18 exhaustion-like changes in Th1 cells. *Card9*-deficient macrophages expressed higher levels of
19 immune checkpoint ligands and secreted increased amounts of IL-10 and TGF- β , suggesting a
20 potential mechanism by which they contribute to Th1 cell dysfunction(41, 45, 46). Further
21 investigation is required to elucidate the underlying mechanisms and the impact of exhausted T cells
22 on antifungal immunity.

1 The regulatory mechanisms governing TREM2 expression remain unclear. Previous research
2 has shown that pro-inflammatory stimuli, such as LPS and IFN- γ , can downregulate TREM2
3 expression in macrophages (60). This likely results from the activation of the NF- κ B signaling
4 pathway, which promotes miR-34a expression, binding and suppressing the transcriptional activity
5 of TREM2 (61). This study elucidated the potential mechanism by which CARD9 regulates the
6 expression of TREM2 in macrophages upon fungal stimulation. We provided evidence that CARD9
7 likely controls TREM2 expression by regulating the balance between NF- κ B/P65 and the CREB-
8 C/EBP β signaling pathway. CARD9 deficiency appeared to induce higher activation of CREB-
9 C/EBP β signaling, which positively regulated the transcriptional programming that drives the anti-
10 inflammatory TREM2^{high} macrophage phenotype. This insight into CARD9-dependent regulation
11 of TREM2 expression in macrophages advances our understanding of how CARD9 orchestrates the
12 local immune response against fungal infections.

13 The findings suggest that targeting TREM2 may enhance antifungal immunity, particularly in
14 CARD9-deficient hosts. TREM2 has been identified as an important target for treating
15 neurodegenerative and infectious diseases and cancer immunotherapy (19). Previous studies have
16 shown that TREM2 knockdown promotes the clearance of bacterial infections and improves T cell
17 responses in cancer immunotherapy (62, 63). In this study, we demonstrated that modulation of
18 TREM2 partially corrected the anti-inflammatory phenotype of CARD9-deficient macrophages in
19 response to fungal stimulation, enhancing their fungicidal activity and ROS generation. Furthermore,
20 this intervention also alleviated exhaustion-like changes observed in Th cells, delaying infection
21 progression in *Card9*^{-/-} mice with dematiaceous fungal infection. These findings highlight the
22 potential of exploiting the TREM2 pathway as an adjunct immunotherapeutic strategy against

1 CARD9-related phaeohyphomycosis. Further investigations are needed to fully elucidate the
2 underlying mechanisms and assess the feasibility of developing TREM2-targeted therapies for
3 clinical management. While the present findings provide valuable insights into the host response to
4 phaeohyphomycosis, it is acknowledged that different fungal pathogens and different infection
5 routes may trigger distinct immune responses and regulatory mechanisms. Therefore, broader
6 implications of TREM2^{high} macrophages in various fungal infections demand further elucidation.

7 In conclusion, this study unveils the mechanism by which CARD9 regulates macrophage
8 phenotype and antifungal function by balancing the CREB-C/EBP β /NF- κ B signaling pathway.
9 Furthermore, we demonstrated the detrimental impact of TREM2^{high} macrophages on host
10 antifungal innate and adaptive immunity, highlighting their potential as therapeutic targets.

11

1 **Methods**

2 **Sex as a biological variable**

3 In mouse studies, mostly male mice were used, with sex and age matched across different groups.

4 For human studies, data were collected from both men and women.

5

6 **Construction of subcutaneous dematiaceous fungal infection model**

7 WT and *Card9*^{-/-} mice were injected subcutaneously in both hind footpads with 100 μL of viable *P.*

8 *verrucosa* (1×10⁸ particles/mL). Starting on day 3 post-infection, mice received intraperitoneal

9 injections of an anti-TREM2 antibody (50 μg per injection, twice weekly). Fungal burden in the

10 infected footpads was determined by plating serially diluted footpad homogenates on Sabouraud's

11 agar (BD Biosciences).

12

13 **Anti-Trem2 Antibody Expression and Purification**

14 The anti-Trem2 antibody (Clone #37012) was constructed and expressed in house. For protein

15 expression, plasmids were mixed with PEI MAX in Freestyle 293 medium at a mass ratio of 1:4.

16 The mixture was used to transiently co-transfect human embryonic kidney (HEK) 293F cells. After

17 6 days of transfection, the supernatant was collected and passed through a 0.22 μM filter. The protein

18 was purified by protein A–Sepharose column according to the manual (Repligen Corporation) and

19 analyzed by reducing and non-reducing SDS-PAGE.

20

21 **Single-cell preparation from skin tissue**

22 Skin biopsy specimens were disassociated using Dispase II (Sigma-Aldrich) to separate the

1 epidermis and dermis. The minced epidermis was further digested with 0.25% Trypsin-EDTA
2 (Gibco) for 30 min and filtered with a 70 µm cell strainer (Falcon). The dermis was digested with 1
3 mg/mL Collagenase P (Sigma-Aldrich) and 100 µg/mL DNase I (Sigma-Aldrich) for 50 min and
4 filtered using a 70 µm cell strainer (Falcon). Barcode labeling of single cells and library construction
5 were performed using a 10× chromium system (10× genomics). The constructed library was
6 sequenced using the Illumina NovaSeq 6000 system.

7

8 **Calculating cell state scores**

9 To elucidate the functional states and underlying biological processes of the cells within our
10 integrated single-cell RNA-sequencing dataset, we leveraged the AddModuleScore function in the
11 Seurat package. AddModuleScore calculates the score for each cell based on the expression of
12 predefined genes, effectively summarizing the activity of the pathway or module within that cell.
13 The predefined gene sets utilized in this study have been specifically annotated in relevant sections
14 within the text.

15

16 **Cell-cell contact analysis**

17 CellChat (v1.6.0) was used to explore communication networks among cell populations, focusing
18 on known ligand-receptor pairs. This analysis aimed to uncover the signaling pathways that mediate
19 interactions between cells, gaining insights into the regulatory mechanisms that underpin cellular
20 cooperation and coordination in our system of interest (64).

21

22 **Detection of ROS production**

1 We measured ROS production as previously described (12). Briefly, BMDM cells were washed with
2 PBS twice and incubated with serum-free DMEM containing 10 μ M DCFH-DA at 37 °C for 30 min.
3 The cells were gently washed thrice and infected with heat-killed *P. verrucosa* (MOI=10) at different
4 time points. The relative amount of ROS generated was detected using a BD FACS flow cytometer,
5 and the mean fluorescence intensity (MFI) in the FITC channel was calculated using Flowjo 10.4
6 software.

7

8 **Statistics**

9 Data were analyzed using GraphPad Prism 9.0 software and are presented as mean \pm SD.
10 Comparisons between the two groups were performed using a two-tailed Student's t-test or pairwise
11 Wilcoxon rank sum test. For comparisons among multiple groups, one-way ANOVA followed by
12 Tukey's post-hoc test was used to determine the statistical significance. Two-way ANOVA was
13 performed to assess the differences in footpad swelling changes over time between the two groups.
14 Statistical significance was determined based on *P* values. n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$,
15 *** $P < 0.001$, **** $P < 0.0001$.

16

17 **Study approval**

18 All mouse experiments were conducted in accordance with the guidelines of the Institutional Ethics
19 Committee of Peking University First Hospital. All patients provided written informed consent
20 before participation.

21

22 **Data availability**

1 The raw scRNA-Seq data reported in this paper have been deposited in the Genome Sequence
2 Archive in National genomics Data Center, China National center for Bioinformatics/Beijing
3 Institution of Genomics, Chinese Academy of Sciences (accession no. CRA028974
4 <https://ngdc.cncb.ac.cn/gsa/browse/CRA028974> and HRA012858 [https://ngdc.cncb.ac.cn/gsa-](https://ngdc.cncb.ac.cn/gsa-human/browse/HRA012858)
5 [human/browse/HRA012858](https://ngdc.cncb.ac.cn/gsa-human/browse/HRA012858)). Values for all data points in graphs are reported in the Supporting
6 Data Values file.

7 Additional details on methods can be found in the Supplemental Methods.

8

9 **Author Contributions**

10 XW, FB, and WW conceptualized this study. LZ, YZ, WL, HJ, KL, YM, and WL conducted the
11 experiments and acquired data. LZ and ZT analyzed the data and wrote the manuscript. XW, FB,
12 and WW edited the manuscript. LY provided reagents for this study. YF and RL provided guidance
13 for this study. RL and XW provided funding.

14

15 **Acknowledgments**

16 This work was supported by the National Key Research and Development Program of China
17 (2022YFC2504800, 2022YFC2504602), the National Natural Science Foundation of China
18 (82273543, 82030095, 82241230, 82341007), Beijing Nova Program (20230484339), the National
19 Science Fund for Distinguished Young Scholars (T2125002), and the Beijing Natural Science
20 Foundation (Z220014).

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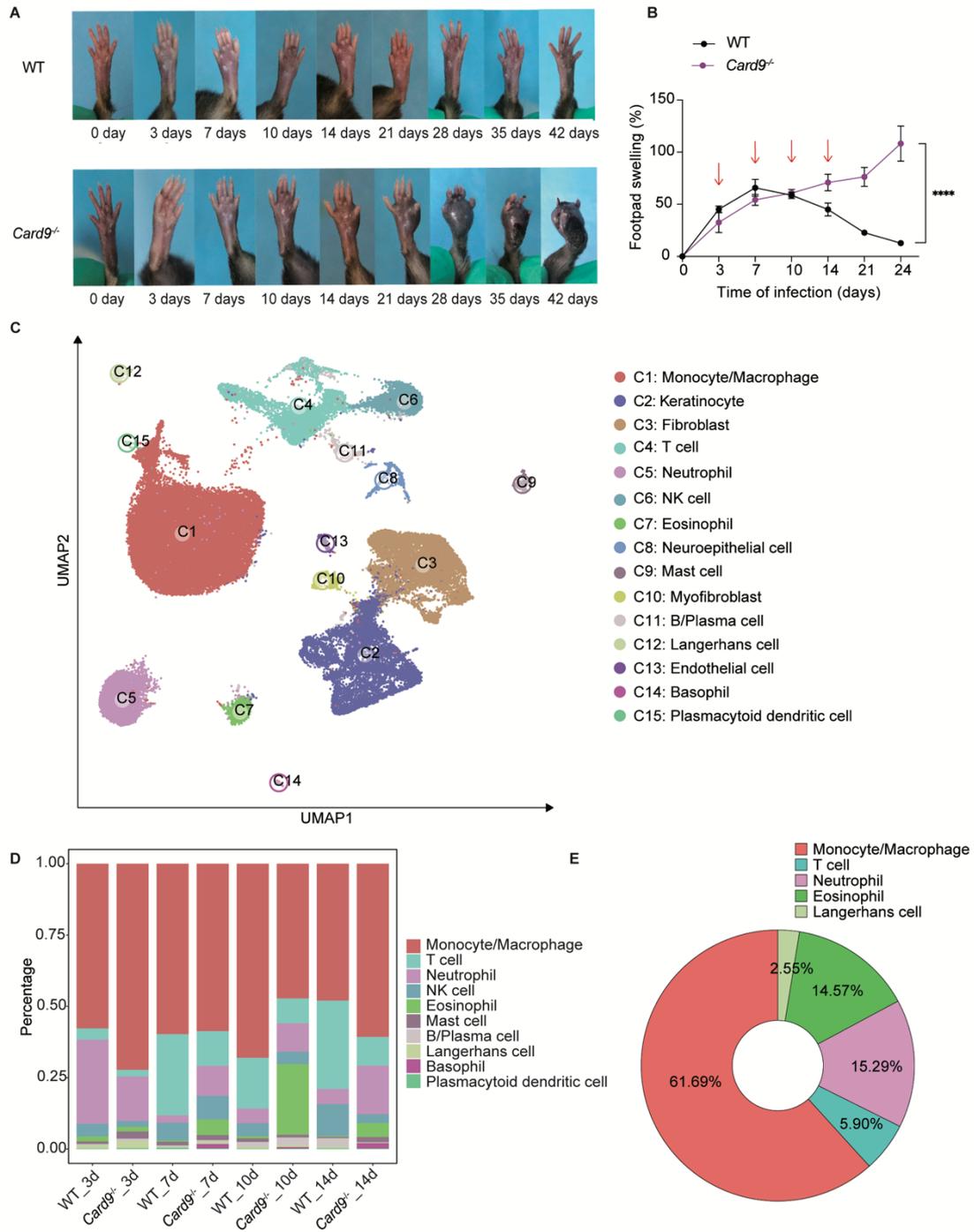
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1 **Figure legends**



2

3 **Figure 1. CARD9 is necessary for defense against subcutaneous dematiaceous fungal infection**

4 (A) Natural course of subcutaneous infection with *P. verrucosa* in WT and *Card9*^{-/-} mice.

5 (B) Footpad swelling of *P. verrucosa*-infected WT and *Card9*^{-/-} mice at different time points after

1 infection (n = 3).

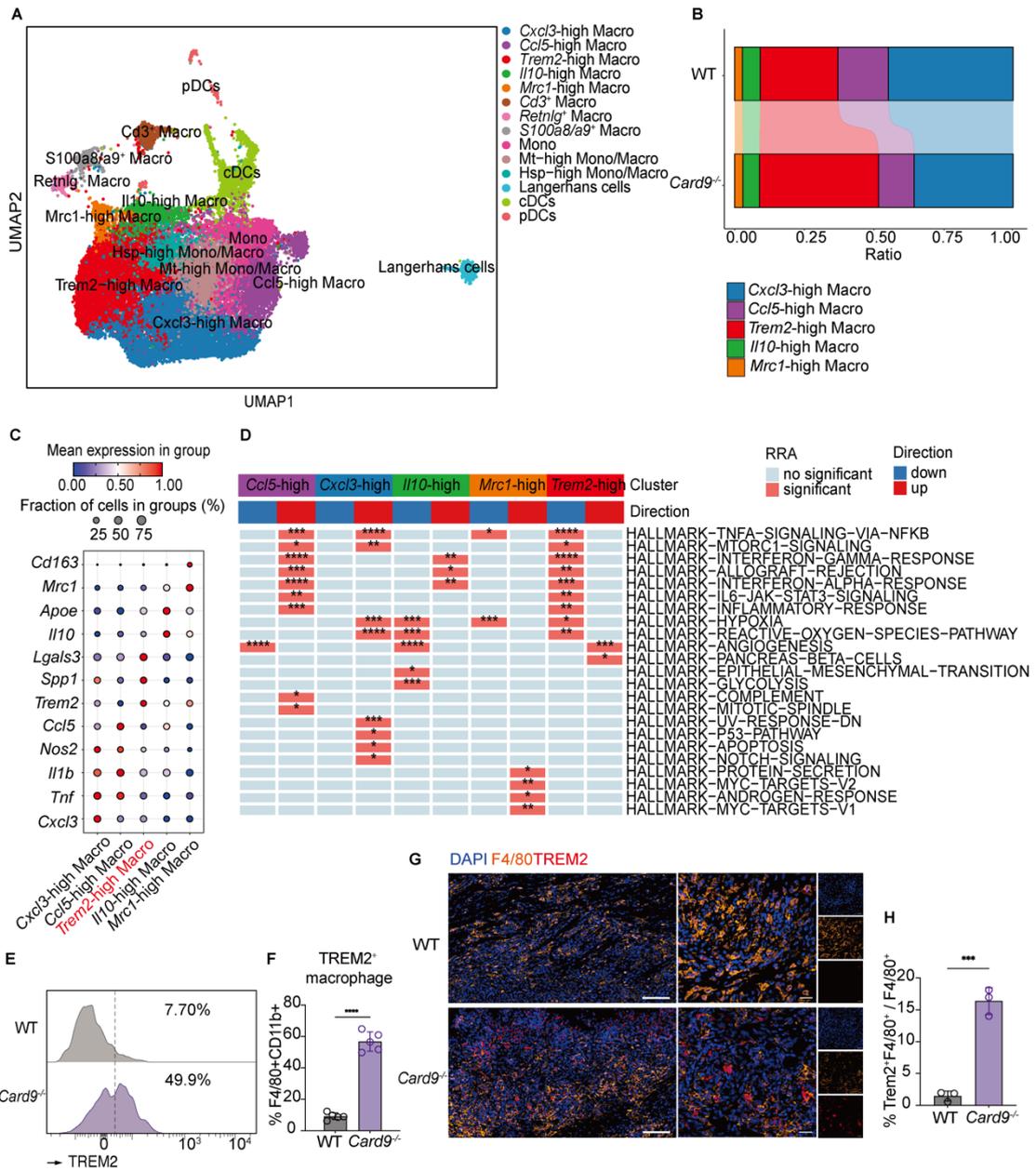
2 (C) The UMAP plot presents the projection of 59,396 high-quality cells from eight single-cell RNA
3 sequencing samples, comprising four samples each from WT and *Card9*^{-/-} groups. Each point on the
4 plot represented a single cell, with colors varying according to distinct cell types.

5 (D) The stacked bar chart showed the percentage distribution of ten immune cell types across all
6 samples. The colors representing each cell type were consistent with those shown in Figure 1C.

7 (E) The pie chart depicted the distribution of *Card9*⁺ cells among immune cell subsets within lesional
8 skin. Data were integrated from all samples.

9 Data are representative of three independent experiments and are shown as the mean ± SD.

10 *****P* < 0.0001, by two-way ANOVA test (B).



1

2 **Figure 2. TREM2^{high} macrophages display anti-inflammatory signatures and are increased in**
 3 **Card9^{-/-} mice**

4 (A) UMAP projection of myeloid cells. Each dot represents a single cell, with colors varying
 5 according to distinct cell subpopulations.

6 (B) The Sankey diagram illustrates the proportional differences between the major macrophage

1 subpopulations across the two groups.

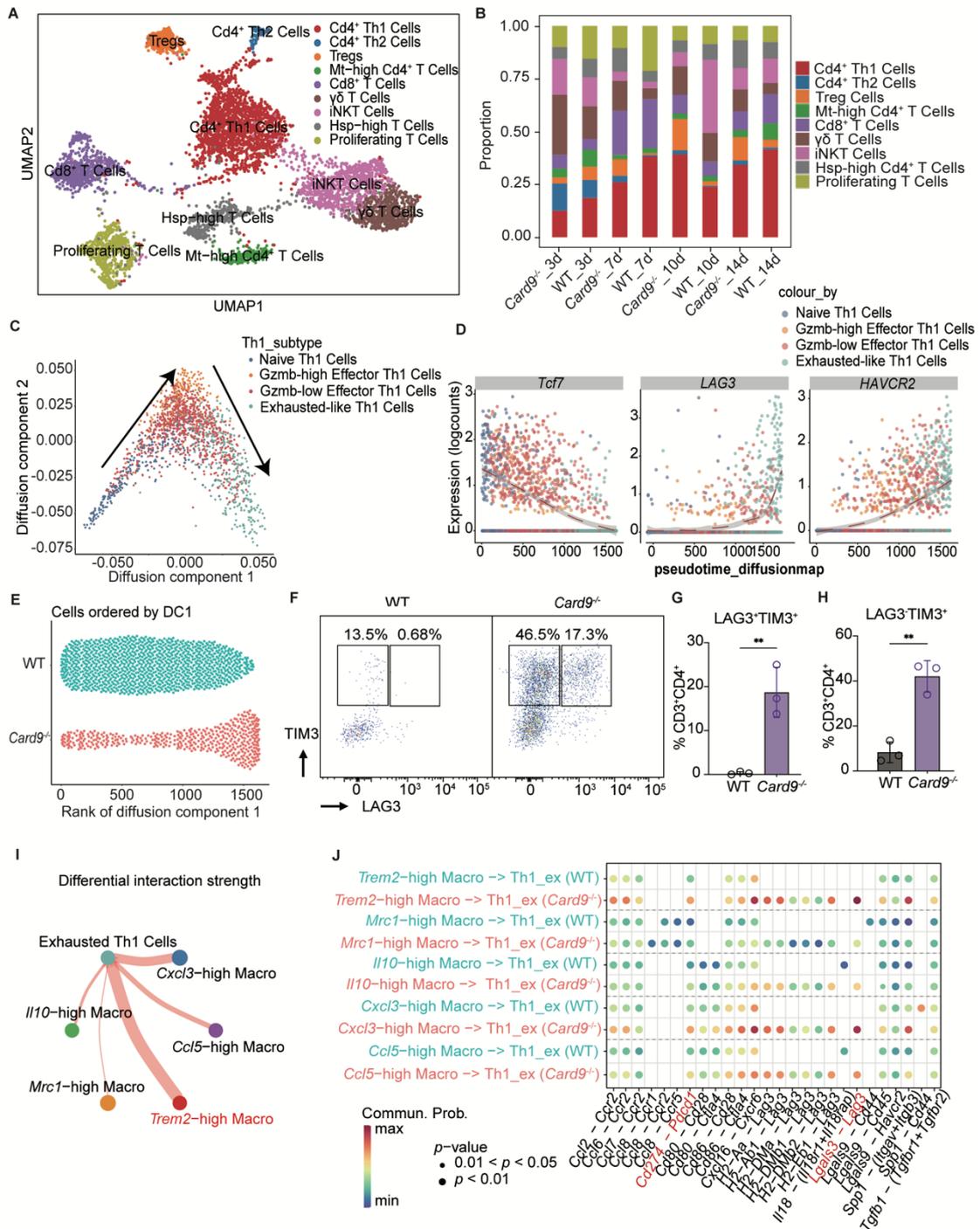
2 (C) Bubble chart shows the mean relative expression of signature genes across the major
3 macrophage subpopulations.

4 (D) Heatmap illustrates the differences in HALLMARK gene set scores among the major
5 macrophage subpopulations, with scores calculated via the “irGSEA” R package
6 (<https://github.com/chuiqin/irGSEA/>). Only gene set scores exhibiting differences across
7 subpopulations were presented.

8 (E and F) Representative flow cytometry histogram plots for TREM2 staining (F) and frequency of
9 TREM2⁺ macrophage subsets (G) in murine footpad lesions at day 10 post-infection. One data point
10 denotes a result from one mouse (n = 5).

11 (G and H) Staining of TREM2⁺ macrophages (TREM2 and F4/80) in murine lesions. Scale bars,
12 100 μm (left) and 20 μm (right). The bar plots show the quantification results (I). One data point
13 represents the statistical result of one field of view (n = 3 fields analyzed per condition).

14 Data are shown as the mean ± SD. *** $P < 0.001$, **** $P < 0.0001$, by two-tailed Student’s t test (G
15 and I).



1 (C) Diffusion map illustrates the developmental trajectory of Th1 cells, with the direction of
2 development indicated by arrows. This process was implemented by using the R package destiny
3 (<https://github.com/theislab/destiny>).

4 (D) Scatter plots show the expression of naive and exhaustion markers throughout the pseudotime
5 of the Th1 cell development process. The dashed line represented the fitted trend of changes. Each
6 point represented a Th1 cell, with its color corresponding to that in Figure 3C.

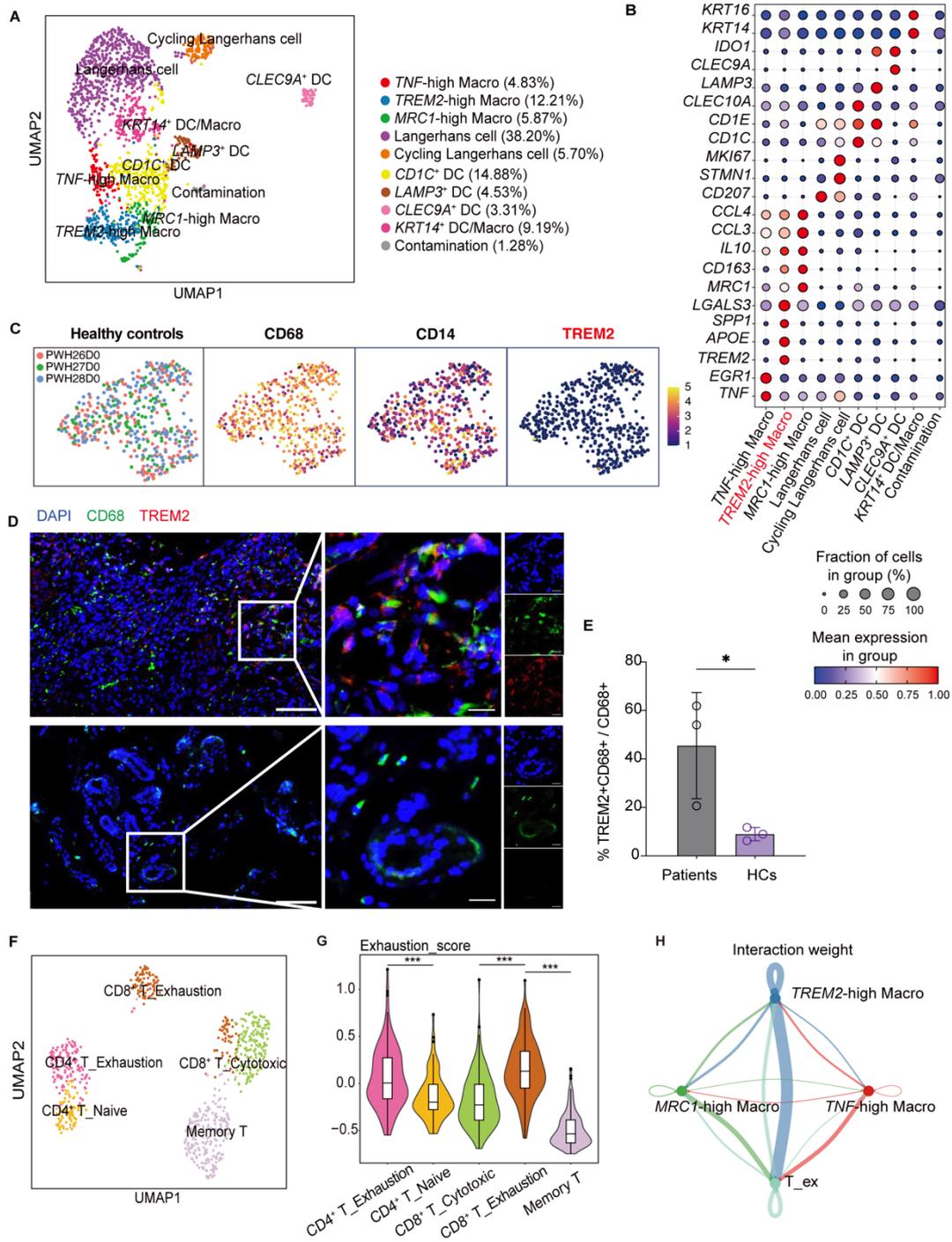
7 (E) Scatter plot shows the distribution of Th1 cells in the WT group and the *Card9*^{-/-} group. Each
8 point represented a Th1 cell, with its x-axis corresponding to the ordinal value of Diffusion
9 Component 1 as arranged from smallest to largest in Figure S3C.

10 (F–H) Representative flow cytometry plots for TIM3 and LAG3 staining and frequency of
11 LAG3⁺TIM3⁺CD4⁺T subsets (G) and LAG3⁻TIM3⁺CD4⁺T subsets (H) in murine footpad lesion at
12 day 10 post-infection. One data point denotes a result from one mouse.

13 (I) The circle plot demonstrates the enhanced interaction strength between the major macrophage
14 subpopulations and exhaustion-like Th1 cells in the *Card9*^{-/-} group.

15 (J) The bubble plot shows the main ligand-receptor pair between the major macrophage
16 subpopulations and exhaustion-like Th1 cells in WT and *Card9*^{-/-} group.

17 Data are shown as the mean ± SD. ***P* < 0.01, ****P* < 0.001, by two-tailed Student's t test (G and
18 H), and pairwise Wilcoxon rank sum test (J).



1

2 **Figure 4. Anti-inflammatory TREM2^{high} macrophages in lesions of CARD9-deficient patient**
 3 **with phaeohyphomycosis**

4 (A) UMAP projection of macrophages and dendritic cells in the CARD9-deficient patient. Each

5 point represents an individual cell, with the proportion of each subset within the total cell population

1 annotated in the graph.

2 (B) Bubble chart shows the mean relative expression of signature genes across the macrophage and
3 dendritic cell subsets.

4 (C) UMAP plot shows the expression of *TREM2* in macrophages within skin tissues obtained from
5 healthy subjects (n = 3).

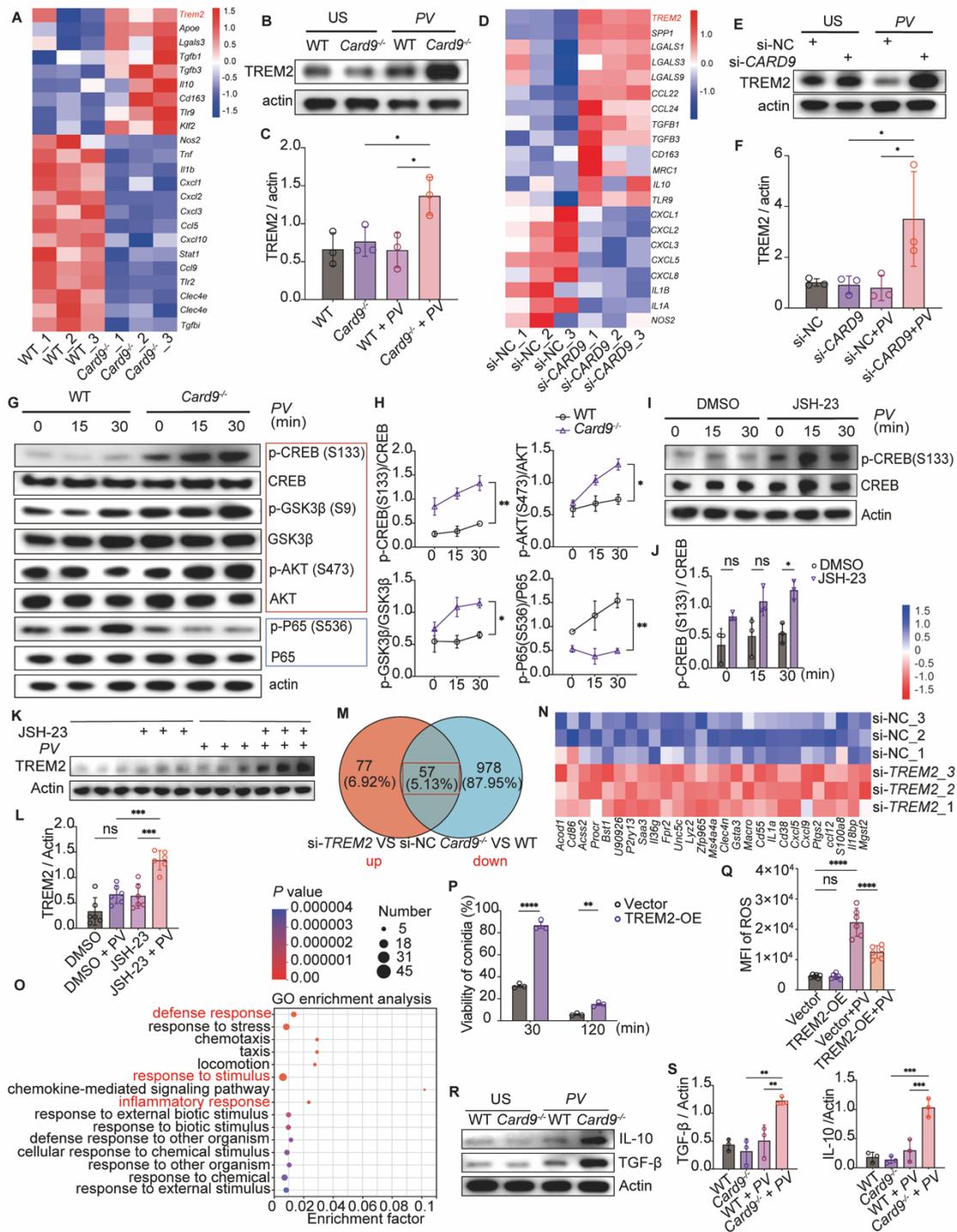
6 (D and E) Representative staining of *TREM2*⁺ macrophages (*TREM2* and *CD68*) in lesions of
7 *CARD9*-deficient patients and controls. Scale bars, 100 μm (left) and 20 μm (right). The bar plots
8 show the quantification results (E). One data point represents the statistical result of one sample (n
9 = 3).

10 (F) UMAP projection displays the cellular distribution of *CD4*⁺T subset, *CD8*⁺T_*GZMK* subset,
11 *CD8*⁺T_*HAVCR2* subset, and Memory T cell subset as depicted in Figure S2A, which were
12 categorized into Naive, Cytotoxic, and Exhaustion states based on the expression of marker genes.

13 (G) Violin plots show the Exhaustion score (defined by the five genes: "*LAG3*", "*TIGIT*", "*PDCDI*",
14 "*CTLA4*", and "*HAVCR2*") of five T cell subsets. Box plots overlaid on the violins depicted the
15 interquartile range and median score for each subset.

16 (H) Bubble chart shows the interactions between the major macrophage subsets and exhaustion-like
17 Th1 cells in the *CARD9*-deficient patient.

18 Data are shown as the mean ± SD. **P* < 0.05, ****P* < 0.001, by two-tailed Student's t test (E), by
19 one-way ANOVA with Tukey's test (G).



1

2 **Figure 5. CARD9 deficiency induces higher TREM2 expression in macrophages and impairs**

3 **antifungal infection**

4 (A–F) Heatmap of selected gene expression from RNA-seq and Western blot with densitometric

5 analysis of TREM2 in BMDMs (A–C) and THP-1 cells (D–F) stimulated with *P. verrucosa* for 24

1 h (n = 3).

2 (G and H) Western blot (G) and densitometric (H) analysis of phosphorylated and total P65, AKT,
3 GSK3 β , and CREB in BMDMs stimulated with *P. verrucosa*.

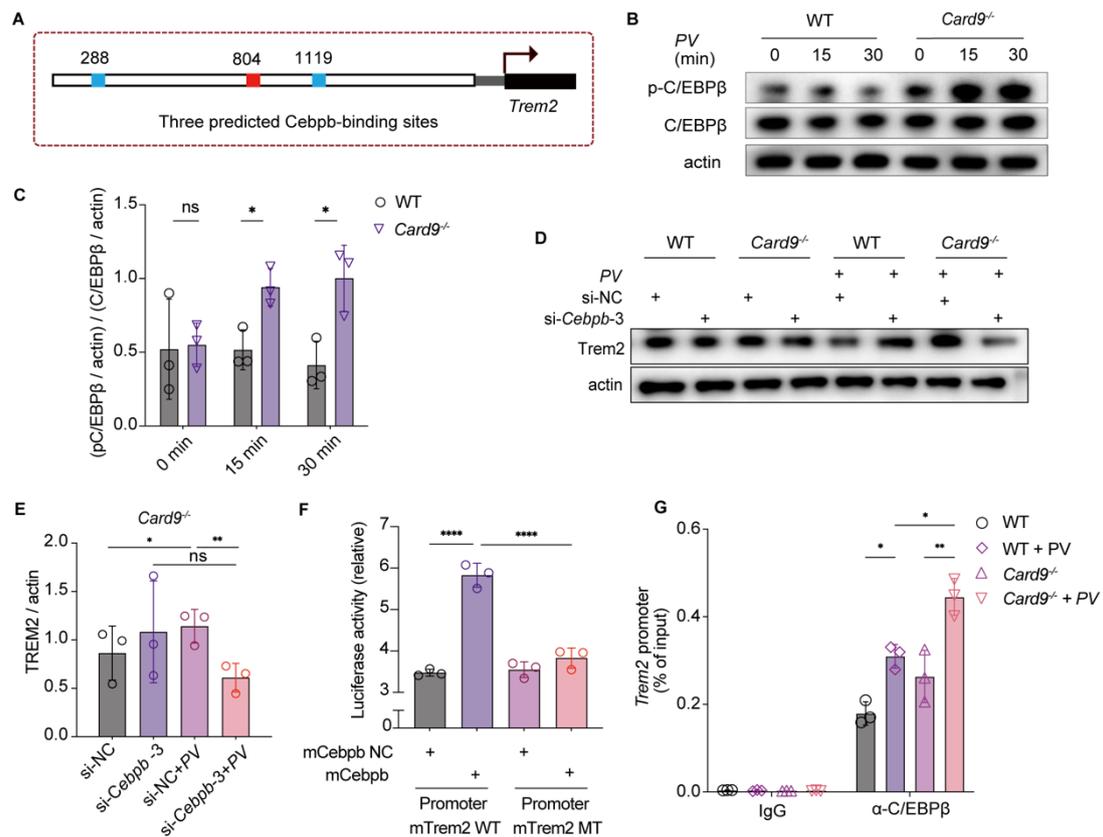
4 (I–L) Western blot and densitometric analysis of phosphorylated CREB (I and J) and TREM2 (K
5 and L) in WT BMDMs with or without JSH-23 pretreatment, following *P. verrucosa* stimulation.

6 (M–O) BMDMs were transfected with si-NC or si-TREM2 and stimulated with *P. verrucosa* for 24
7 h. Venn diagram (M) and heatmap (N) show the overlap between genes downregulated in *Card9*^{-/-}
8 si-NC versus WT si-NC and those upregulated in *Card9*^{-/-} si-TREM2 versus *Card9*^{-/-} si-NC. Bubble
9 plot shows GO enrichment of the overlap genes in *Card9*^{-/-} si-TREM2 BMDMs (O).

10 (P and Q) Killing efficacy analysis (P) and ROS production of TREM2-overexpressing RAW 264.7
11 cells and controls with *P. verrucosa* stimulation for 60 min (Q).

12 (R and S) Western blot (R) and densitometric (S) analysis of IL-10 and TGF- β in BMDMs
13 stimulated with *P. verrucosa* for 72 h.

14 In A, D, and M, columns represent replicates from independent culture wells (n = 3). In C, F, H, J,
15 L, and S, each point represents an independent replicate. Data are shown as mean \pm SD. * P < 0.05,
16 ** P < 0.01, *** P < 0.001, **** P < 0.0001, by one-way ANOVA with Tukey's multiple-comparison
17 test (C, F, L, Q, and S), two-way ANOVA test (H), and multiple unpaired t-tests with Holm-Šídák
18 correction (J and P). All stimulations used heat-killed *P. verrucosa* at MOI = 10. US, unstimulated;
19 PV, *Phialophora verrucosa*; NC, negative control; OE, over-expressing.



1

2 **Figure 6. CARD9 negatively regulates *Trem2* expression by activating C/EBPβ**

3 (A) Promoter region of wild-type *Trem2*, showing three predicted C/EBPβ-binding sites at positions.

4 (B and C) Western blot (K) and densitometric (L) analysis phosphorylated (p-) and total C/EBPβ

5 (left margin), in BMDMs isolated from WT and *Card9*^{-/-} mice and stimulated for 0–30 min (above

6 lanes) with heat-killed *P. verrucosa* conidia (MOI=10). Each data point represents an independent

7 experimental replicate (n = 3).

8 (D and E) Knockdown of endogenous C/EBPβ by RNA interference in BMDMs, which were

9 transfected with siRNA against murine C/EBPβ and nontargeting control siRNA using

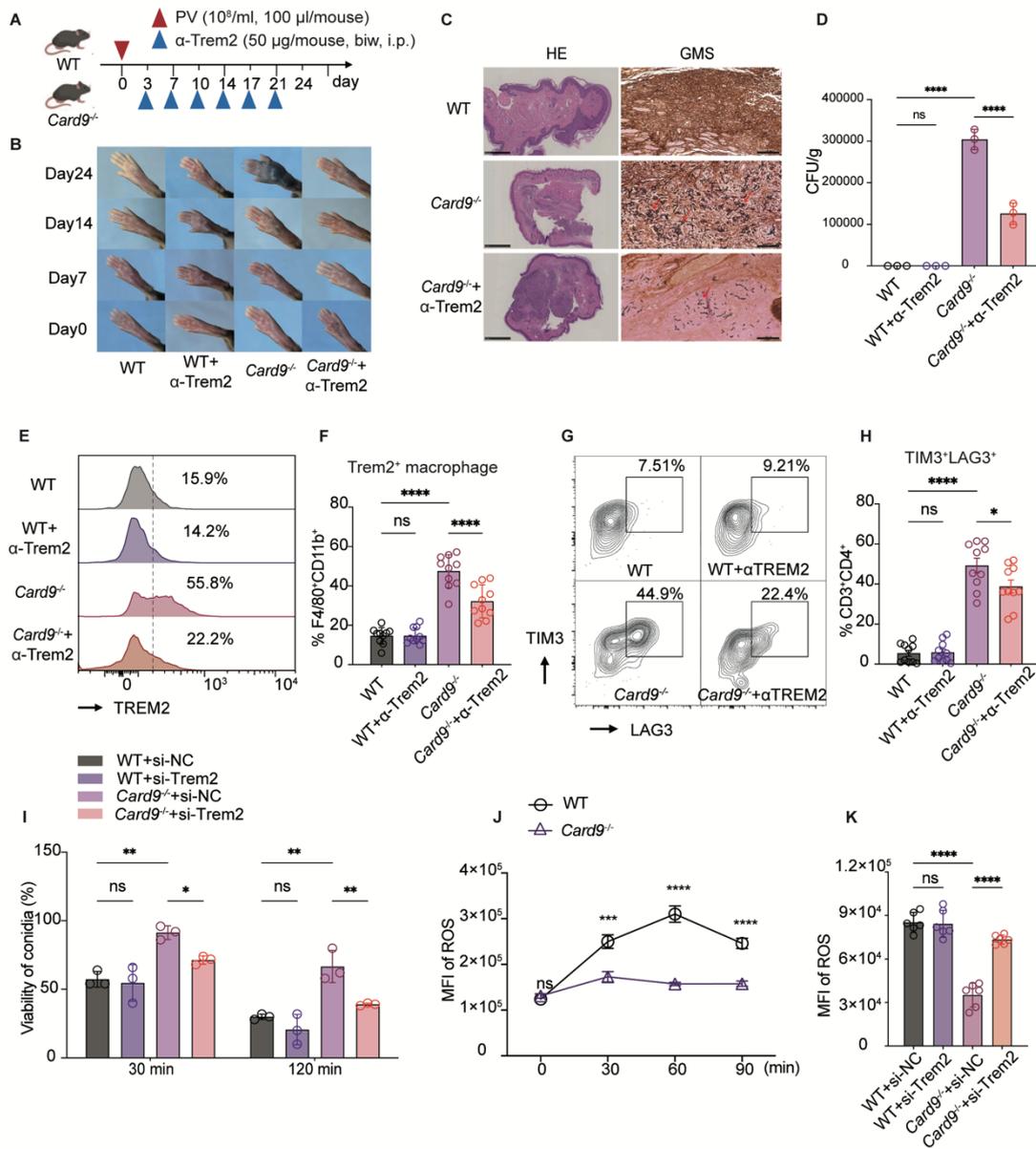
10 Lipofectamine 3000 transfection reagent (Thermo Fisher). Cells were cultured for 48 h after

1 transfection and then stimulated with heat-killed *P. verrucosa* conidia (MOI=10) for 24 h. Cell
2 lysates were subjected to western blot analysis using indicated antibodies (M) and then quantified
3 using densitometric analysis in *Card9*^{-/-} group (E). Each data point represents an independent
4 experimental replicate (n = 3).

5 (F) Firefly luciferase activity in HEK293T cells co-transfected with constructs for the
6 overexpression of Cebpb and a construct containing various *Trem2* promoter-driven firefly
7 luciferase constructs together with an EF1 α promoter-driven renilla luciferase reporter; results were
8 normalized to those of renilla luciferase. Ctr, control construct lacking Cebpb co-transfected with a
9 construct containing the *Trem2* promoter.

10 (G) Chromatin immunoprecipitation (with control IgG or anti-Cebpb) and PCR analysis of the
11 binding of Cebpb to the *Trem2* promoter in BMDMs obtained from WT mice and *Card9*^{-/-} mice and
12 left unstimulated or challenged for 4 h *in vitro* with heat-killed *P. verrucosa* spores (MOI=10).

13 Data are shown as the mean \pm SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.001, by multiple
14 unpaired t-tests with Holm-Šídák correction (C), one-way ANOVA with Tukey's multiple-
15 comparison test (E–G). PV, *Phialophora verrucosa*; NC, negative control; mTrem2, mouse Trem2;
16 WT, wild-type; MT, mutant-type.



1

2 **Figure 7 Anti-TREM2 antibody improves the antifungal immune response *in vivo* and *in vitro***

3 (A and B) WT and *Card9*^{-/-} mice were subcutaneously injected with *P. verrucosa* and treated

4 intraperitoneally with PBS or anti-TREM2 antibody. Experimental scheme (A) and natural course

5 (B) of infection.

1 (C) Histopathology of H&E-, Grocott's Methenamine Silver (GMS) footpad from infected mice at
2 day 14 after infection. Scale bars, 1 mm (H&E) and 100 μ m (GMS). Arrows indicate fungal yeast
3 and hyphae.

4 (D) Fungal burden of footpad from WT and *Card9*^{-/-} mice on day 14 after infection.

5 (E and F) Representative flow cytometry histogram plots for TREM2 staining (E) and frequency of
6 TREM2⁺ macrophage subsets (F) in murine lesions. One data point denotes a result from one mouse.
7 (n = 10, from three independent experiments).

8 (G and H) Representative flow cytometry contour plots for TIM3 and LAG3 staining (G) and
9 frequency of TIM3⁺ LAG3⁺CD4⁺T subsets (H) in murine lesions. One data point denotes a result
10 from one mouse. (n = 10, from three independent experiments).

11 (I-K) Knockdown of endogenous TREM2 by RNA interference in BMDMs. Cells were cultured for
12 48 h after transfection and then stimulated with heat-killed *P. verrucosa* spores (MOI=10) for the
13 indicated times. Killing efficacy analysis of BMDMs of *Card9*^{-/-} mice (I). Total ROS production of
14 WT and *Card9*^{-/-} BMDMs at the indicated time point was measured by the Reactive Oxygen Assay
15 kit (Beyotime) (J and K).

16 Data in B, C and I–K are representative of three independent experiments. Data are shown as the
17 mean \pm SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.001, by one-way ANOVA with Tukey's
18 multiple-comparison test (D, F, H, I, and K), two-way ANOVA with Šídák's multiple-comparison
19 test (J). PV, *Phialophora verrucosa*; NC, negative control.