




The pro-apoptotic ARTS protein induces neutrophil apoptosis, efferocytosis, and macrophage reprogramming to promote resolution of inflammation

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Abstract

ARTS (Sept4_i2) is a pro-apoptotic protein and a product of the Sept4 gene. ARTS acts upstream of mitochondria to initiate caspase activation. ARTS induces apoptosis by specifically binding XIAP and allowing de-repression of active caspases required for Mitochondrial Outer Membrane Permeabilization (MOMP). Moreover, ARTS promotes apoptosis by inducing ubiquitin-mediated degradation of both major anti-apoptotic proteins XIAP and Bcl-2. In the resolution phase of inflammation, the infiltrating leukocytes, which execute the acute innate response, undergo apoptosis and are subsequently cleared by phagocytic macrophages (i.e. efferocytosis). In this course, macrophages undergo reprogramming from inflammatory, to anti-inflammatory, and eventually to resolving macrophages that leave the injury sites. Since engulfment of apoptotic leukocytes is a key signaling step in macrophage reprogramming and resolution of inflammation, we hypothesized that a failed apoptosis in leukocytes in vivo would result in an impaired resolution process. To test this hypothesis, we utilized the Sept4/ARTS^{-/-} mice, which exhibit resistance to apoptosis in many cell types. During zymosan A-induced peritonitis, Sept4/ARTS^{-/-} mice exhibited impaired resolution of inflammation, characterized by reduced neutrophil apoptosis, macrophage efferocytosis and expression of pro-resolving mediators. This was associated with increased pro-inflammatory cytokines and reduced anti-inflammatory cytokines, secreted by resolution-phase macrophages. Moreover, ARTS overexpression in leukocytes in vitro promoted an anti-inflammatory behavior. Overall, our results suggest that ARTS is a key master-regulator necessary for neutrophil apoptosis, macrophage efferocytosis and reprogramming to the pro-resolving phenotype during the resolution of inflammation.

Keywords Resolution of inflammation · Neutrophil apoptosis · Macrophage reprogramming · Efferocytosis · ARTS

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Introduction

Apoptosis or programmed cell death is an essential process for normal development and tissue homeostasis. Abnormal regulation of this process is associated with a wide variety of human diseases, including immunological and developmental disorders, neurodegeneration and cancer [1–3]. Apoptosis can be initiated by both extrinsic and intrinsic signals centered in and from the mitochondria [4, 5]. Both pathways are executed by activating caspases (cysteine-aspartic proteases) through cleavage from their inactive zymogens [6–8]. Apoptotic caspases are organized into “initiator caspases” (caspase-2, -8, -9, and -10) and effector caspases (caspase-3, -7, and -6) [8–10]. Caspases 8 and 10 are cleaved primarily in response to extrinsic signals, while caspase-9 is activated in the intrinsic mitochondrial

pathway. These enzymes act in a cascade that culminates in cleavage of multiple cellular proteins, resulting in the death of the cell [7]. In living cells, caspases are kept in check by inhibitors of apoptosis (IAP) proteins [11, 12]. The best studied IAP is XIAP, which is the only IAP that can directly binds and inhibit caspases [13–15]. Notably, XIAP contains a RING domain responsible for E3-ligase activity [16–19]. IAPs are negatively regulated by IAP-antagonist proteins, such as Smac (second mitochondrial-derived activator of caspases/Diablo, OMI/HTRA2, XAF1 and ARTS (Apoptosis Related protein in the TGF- β Signaling pathway) [20–27]. ARTS (Sept4_i2) (Apoptosis-Related protein in the TGF β Signaling pathway) is a splice variant derived from the Sept4 (Septin 4) gene, and the only splice variant that functions as a pro-apoptotic protein [28]. Although ARTS was originally discovered in cells induced for apoptosis by TGF- β [24], it was later found to act downstream of all apoptosis stimuli tested, such as treatment with STS (Staurosporine), Etoposide, Arabinoside (Ara-c), Nocadsole, UV radiation, TNF- α , etc [24, 27, 29–31]. ARTS acts upstream of mitochondria to initiate caspase activation [29, 32, 33]. ARTS is localized at the outer membrane of the mitochondria [29]. Upon apoptotic stimuli, ARTS rapidly translocates to the cytosol in a caspase-independent manner and antagonizes XIAP [27, 29]. ARTS binds directly to the XIAP/BIR3 domain and promotes the auto-ubiquitylation and degradation of XIAP, in addition to serving as an adaptor bringing the E3-ligase Siah to stimulate the degradation of XIAP [27, 29, 34]. Moreover, ARTS acts as a scaffold by bringing XIAP with its E3-ligase activity, into close proximity with Bcl-2, promoting UPS-mediated-degradation of Bcl-2 [35]. Thus, ARTS functions as a dual antagonist of both XIAP and Bcl-2 to initiate Mitochondrial outer membrane permeabilization (MOMP) and apoptosis. Furthermore, the translocation of ARTS from the MOM to the cytosol precedes MOMP and the release of Cyto c and Smac, and is required for it [29, 35]. Human and mice studies have shown that ARTS functions as a potent tumor suppressor protein. ARTS expression is lost in more than 70% of Acute Lymphoblastic Leukemia (ALL) patients [31], in 50% of lymphoma patients [36], and in a significant fraction of hepatocellular carcinoma (HCC) patients (S. Larisch and H. Steller, unpublished data). Studies using Sept4/ARTS^{null} mice showed that ARTS is a physiological antagonist of XIAP in vivo. In particular, Sept4/ARTS^{null} mice have increased numbers of hematopoietic stem and progenitor cells (HSPCs) which are resistant to apoptosis [36]. Whole-body deletion of Sept4/ARTS equips the Intestinal stem cells (ISCs) niche with increased resistance against apoptosis [37]. In addition, Sept4/ARTS deficient mice have elevated numbers of hair follicle stem cells (HFSCs) that are protected against apoptosis and display marked improvement in wound healing and regeneration of hair follicles [38]. These mice exhibit spontaneous

accelerated tumor development and elevated XIAP levels [36–39]. This suggests that the pro-apoptotic function of ARTS as an XIAP antagonist and its function in stem cells may serve to inhibit the emergence of cancer [39]. Moreover, the resistance of Sept4/ARTS^{null} HSPCs to apoptosis and the cell-autonomous lymphoproliferation is suppressed by the loss of XIAP function in Sept4/ARTS/XIAP double-knockout mice [36]. These results demonstrate the important physiological role of ARTS in regulating apoptosis and tumor suppressor in vivo through its role as a specific XIAP-antagonist [33].

The active termination of an inflammatory process is termed the resolution of inflammation; distinct molecular and cellular events are triggered to ensure the eventual complete resolution that is essential to restore tissue homeostasis [40, 41]. To achieve that homeostasis, polymorphonuclear cells (PMNs), typically short-lived neutrophils that infiltrate sites of inflammation to fight pathogens, must be eliminated. Thus, apoptosis of PMNs and the subsequent clearance by macrophages plays a major role in initiating the resolution of the acute inflammatory response [42]. Removal of these cells occurs rapidly and without induction of a pro-inflammatory response. The engulfment of apoptotic cells, termed efferocytosis, drives the reprogramming of the engulfing macrophages to anti-inflammatory/repairative macrophages, which secrete lower levels of pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF α), and higher levels of anti-inflammatory cytokines, such as transforming growth factor- β (TGF β), and in some cases IL-10 [42–44]. This reprogramming is also known as type I (M1, pro-inflammatory) to Type II (M2c, anti-inflammatory/deactivated) macrophage conversion. Reprogramming to M2 macrophages inhibits the expression of cyclooxygenase-2 (COX2), matrix metalloproteinase-9 (MMP-9) and inducible nitric oxide synthase (iNOS), which are key enzymes in some inflammatory cytotoxic response [42–44]. Concomitantly, M2 macrophages stimulate the expression of arginase-1, which competes with iNOS for the same substrates; reactive NO production is prevented thereof [45, 46]. Recent reports further characterized another step in macrophage reprogramming – the conversion to pro-resolving macrophages with unique properties, such as the ability to release immunomodulatory lipid mediators [40, 41, 43, 47]. As compared to peritoneal M1 and M2 macrophages, pro-resolving macrophages are identified by a lower surface expression of CD11b and F4/80, reduced expression of the respective M1 and M2 markers iNOS and arginase 1, and increased expression of the enzyme implicated in the synthesis of specialized pro-resolving lipid mediators, 12/15-lipoxygenase (LO) [40, 48, 49]. Eosinophils also take part in the resolution of inflammation by the production of similar “stop signal” mediators [50, 51]. The resolution sequel ends with the departure of the pro-resolving macrophages through the

lymphatic system [40, 43,48]. A failure to complete resolution of acute inflammation may result in chronic inflammation, impaired wound healing that culminates in debilitating tissue fibrosis and organ dysfunction [40, 52–54].

Apoptotic PMNs are key effectors in the resolution of inflammation, through their immunomodulatory properties on ingesting macrophages [55]. These anti-inflammatory mechanisms include inhibition of phagocytic activity in macrophages [40] and release of sphingosine-1-phosphate (S1P) that is capable of suppressing inflammatory macrophages [56, 57]. Another efficient mechanism is the sequestration of chemokines that normally attract inflammatory leukocytes to the inflamed efferent sites, thereby reducing their bioavailability. This is pursued by expression of certain chemokine receptors upon apoptosis—CCR5 and the atypical decoy receptor D6/ACKR2 [58, 59]. D6/ACKR2 exerts different signaling from classical chemokine receptors and hence continuously recycles to the cell surface in a ligand-independent manner [60]. Both receptors are unable to elicit a chemotactic activity in apoptotic leukocytes [58–61], albeit we have shown recently that D6-bound CCL5 on these cells is able to promote the reprogramming of resolution phase macrophages [62]. Taken together, mechanisms triggered by both macrophages and the phagocytosed apoptotic corpses allow a self-limiting and short-lived inflammatory response. In this study, we hypothesized that apoptosis-resistant PMNs would possibly impair resolution of inflammation *in vivo*. For that purpose, we utilized the Sept4/ARTS^{-/-} mice, which were shown to exhibit reduced response / resistance to apoptotic stimuli. Here, we report that the Sept4/ARTS^{-/-} mice exhibit impaired resolution of inflammation, which is characterized by reduced apoptosis in PMN, and consequently reduced efferocytosis and hampered macrophage reprogramming to pro-resolving phenotypes with anti-inflammatory cytokine production.

Materials and methods

Mice

Sept4/ARTS^{-/-} mice were generously provided by Prof. Steller, Rockefeller University, New York city. Interbreeding with C57BL/6 mice gave rise to ARTS^{+/+} (WT), ARTS^{+/-} and ARTS^{-/-} offspring (6–8-week-old; protocol approved by the Committee of Ethics, University of Haifa).

Isolation of resident macrophages

To isolate bone marrow (BM) macrophages, abdomen and hind legs of Sept4/ARTS^{+/+} or Sept4/ARTS^{-/-} mice were sterilized with 70% ethanol. Pelvic and femoral bones were dissected and excess muscle from legs was removed.

Each bone end was cut off and BM was flushed with 1 ml of PBS until bone cavity appeared white. Then, cells were centrifuged at 1200 rpm for 7 min, and the supernatant was discarded. Cell pellet was resuspended in 1% BSA in PBS and macrophages thereof were isolated using EasySep PE selection magnetic beads directed against F4/80, following the manufacturer's instructions (StemCell Technologies). To isolate spleen macrophages, spleens were dissected from the abdominal cavity and meshed against a plastic grid in 1 ml of PBS. The recovered specimen were filtered through 0.45 µm sterile filter, after which the collected cells were centrifuged at 1200 rpm for 7 min. The supernatant was discarded and pellet was treated with 1 ml of RBC lysis buffer for 2 min on ice. Pellet was washed with 10 ml of 1X PBS and then macrophages were isolated as above.

Zymosan A peritonitis

Age-matched WT, Sept4/ARTS^{+/-} or Sept4/ARTS^{-/-} mice were injected intra-peritoneally with zymosan A (Sigma-Aldrich, 1 mg/ml in sterile PBS, 1 ml/mouse). After 24 h or 66 h, mice were euthanized with CO₂, and peritoneal exudates were collected by lavaging with 5 ml of sterile saline.

Flow cytometry

For the determination of leukocyte subtypes and expression of surface markers, exudates were stained with FITC-conjugated anti-mouse Gr-1 or Ly6G (0.5 µg/10⁶ cells), PE-conjugated anti-mouse F4/80 (clone CI:A3-1; 0.2 µg/10⁶ cells), Pacific Blue- or PerCP-conjugated rat anti-mouse Ly6C, APC-conjugated anti-mouse Tim4 or PerCP-conjugated anti-mouse CD11b (clone M1/70;1; 0.2 µg/10⁶ cells); all from BioLegend. Gating strategy to distinguish cell types is depicted in Fig. S1. Propidium Iodide (PI) and Annexin V (2 µl per sample) (BioLegend) were used to identify necrotic and apoptotic cells, respectively. After washing the cells twice with 1% BSA in PBS, the cells were analyzed using FACSCalibur or FACSCantoII (BD Biosciences). Data analysis was performed using the FlowJo software. In addition, eosinophils were identified, and their level of degranulation was determined, using side scatter.

Isolation of murine peritoneal macrophages

Cells were recovered from peritoneal exudates 66 h following zymosan A challenge. Macrophages were labeled with PE-conjugated rat anti-F4/80 and isolated using EasySep-PE selection magnetic beads according to the manufacturer's instructions (Stem-Cell Technologies). The PE negative fraction was designated Macrophage-depleted leukocytes (MDLs).

Neutrophil apoptosis

Peritoneal PMNs from WT or Sept4/ARTS^{-/-} mice at 24 h PPI were isolated using PE-conjugated Gr-1 antibodies with PE selection magnetic beads (StemCell Technologies). PMNs were incubated (10^6 cells/1 ml of culture media) for 24 h and then washed with PBS and resuspended ($2 \times 10^5/100 \mu\text{l}$) in PBS. The cells were stained with AnnexinV-FITC and PI MEBCYTO Apoptosis Kit (MBL Laboratories) and analyzed for live, early and late apoptotic cells by flow cytometry using FACSCanto II.

Apoptotic cell engulfment

Jurkat (human CD4⁺ T cell leukemia cell line) cells were incubated (10^6 cells/ml) with 1 μM staurosporine (Sigma) for 4 h to trigger apoptosis. In some experiments, apoptotic cells were then washed with serum-free media and incubated (10×10^6 cells/ml) with 10 mM CypHer5E Mono NHS Ester (GE Healthcare) for 30 min. Before incubation with macrophages, the apoptotic cells were washed twice with culture medium. Macrophages were recovered from male WT or Sept4/ARTS^{-/-} mice 48 h or 66 h post peritonitis for the ex vivo and in vivo assay of apoptotic cell uptake, respectively. Macrophages were incubated for 4 h with labeled apoptotic cells for ex vivo evaluation or immediately stained for in vivo evaluation of uptake. Unbound cells were washed and macrophages from both assays were transferred to poly-prep slides (Sigma-Aldrich) and fixed with 2% paraformaldehyde. Then, cells were stained with Hoechst 33342 (Invitrogen) and Phalloidin-FITC (Biotium) at room temperature for 20 min and enumerated under a fluorescent microscope (Zeiss). Two areas of two cover slips, each containing at least 50 (overall 200) macrophages were analyzed, and the average number of PMN or Jurkat cells engulfed per macrophage, as well as the number of macrophages with cutoff numbers of engulfed PMN were calculated. Phagocytic index was calculated as previously described [63].

Cell culture

Murine peritoneal macrophages were obtained from mice 66 h post peritonitis. Jurkat cells were used as a model for apoptotic PMNs and THP-1 cells (human acute monocytic leukemia cell line) were used as model for macrophages. All cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 2 μM glutamine, 100 units/ml penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin. Human cell lines were maintained at 37 °C in a 5% CO₂ humidified atmosphere, while primary murine cells were maintained at 37 °C in a 7.5% CO₂ humidified atmosphere. Apoptosis of Jurkat cells was induced using the following methods:

(1) Exposure to UV radiation with a 3 ft. 30 watt UV tube (Airstream S-series Class II Biohazard Safety Cabinet "ESCO®") for 5 min; (2) Incubation with 1 μM staurosporine (STS); (3) Transfection with ARTS-containing plasmids as follows—Freshly cultured Jurkat cells (10^6 cells/ml) were transfected with plasmids encoding c-myc-tagged ARTS vector, according to the jetPEI™ transfection reagent (Polyplus®) protocol as described [64]. THP-1 cells (0.5×10^6 cells /ml) were differentiated to macrophages by incubation with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) for 72 h. Co-culture—transfected Jurkat cells (0.16, 1.5, or 5 million cells) were added to differentiated THP-1 macrophages at 1:3, 3:1, or 10:1 ratios.

Cytokine secretion

Murine peritoneal macrophages (10^6 cells/well) were activated by LPS (1 $\mu\text{g}/\text{ml}$; Sigma Aldrich) for 16 h, while human differentiated THP-1 macrophages (10^6 cells/well) were activated by LPS (500 ng/ml) for 24 h. The cell-free supernatants were collected and the levels of TNF- α , IL-1 β , IL-6, IL-10 and IL-12 were determined by standard ELISA kits, according to the manufacturer's instructions (R&D systems, Minneapolis; BioLegend).

Western Blot

Cells were collected, centrifuged, washed with PBS and lysed in RIPA buffer containing Protease Inhibitors Cocktail (PIC, Sigma-Aldrich). Samples were run by SDS-PAGE (10%) and transferred to a PVDF membrane. The membranes were blotted with antibodies against the following antigens: β -actin, c-Myc, GAPDH and tubulin (Santa-Cruz); arginase-1, iNOS (Abcam); D6 (Abnova); COX2, 12/15-LO, sphingosine kinase (SphK) (Cayman Chemicals); MMP-9 and CCR7 (R&D Systems); H2AX or phospho-H2AK (Calbiochem); Caspase-9 and PARP (Cell Signaling). Anti-CCR2 and anti-CCR5 antibodies were a gift from Dr. Mack (Klinikum der Universitat Regensburg, Regensburg, Germany), and the anti-galectin-1 antibody was a gift from Dr. Rabinovich (Universidad de Buenos Aires, Buenos Aires, Argentina). The membranes were reacted with matching secondary antibodies conjugated with Horseradish Peroxidase (HRP, Jackson Immuno-research laboratories). The membranes were developed with EZ-ECL detection kit (Biological Industries, Beit Haemek) and analyzed using Luminescent Image Analyzer LAS-4000 (Fujifilm Corporation) & "Image Reader LAS-4000" software (Fujifilm Corporation). Densitometry analysis of blot lanes was done using ImageJ 1.50.

Statistical analysis

Experiments were performed at least 3 times with 3–4 replicates of each data point. Results were analyzed by the two-tailed Student's *t*-test, one- or two-way ANOVA, and Pearson correlation. Data are presented as mean \pm SEM. Results were considered statistically significant when $p < 0.05$ (*), 0.01 (**), 0.001 (***)).

Results

Sept4/ARTS^{-/-} mice display an aberrant distribution of peritoneal leukocyte populations and macrophage reprogramming

To determine whether Sept4/ARTS deficiency affect leukocyte numbers at homeostatic conditions, we enumerated the differences in myeloid cell populations between unchallenged male Sept4/ARTS^{-/-} mice and their WT controls. We observed an elevation in the number of bone marrow (BM) neutrophils and Ly6C^{lo} monocytes (Fig. S2a, b), which could be ascribed to lower apoptosis rate. There were no differences in Ly6C^{hi} monocytes, macrophages and other BM-resident leukocytes (Fig. S2b, c and data not shown). Analysis of myeloid cell populations in the spleen and peritoneum indicated no difference in the number of neutrophils, monocytes, resident Tim4⁺ macrophages and other subsets (Fig. S2d–j and data not shown). Overall, there were no major differences between the genotypes in steady state. In order to evaluate whether ARTS-mediated apoptosis in leukocytes affects the resolution of inflammation, we treated male WT, Sept4/ARTS^{+/-} and Sept4/ARTS^{-/-} mice with zymosan A. Zymosan A-induced peritonitis is a well-established model of spontaneously-resolving inflammation [65]. Using this model, we did not observe any difference in the total number of peritoneal leukocytes (Fig. 1a). At 24 h post-injection, neutrophil recruitment is normally at its peak, while at 66 h post-injection, apoptotic neutrophil efferocytosis and clearance are normally observed [55, 59, 65, 66]. As compared to WT mice, Sept4/ARTS^{-/-} mice displayed apoptosis and clearance rates rather than a larger fraction of peritoneal neutrophils at 66 h post induction, but not at 24 h post induction (Fig. 1b). This result suggests that Sept4/ARTS^{-/-} mice exhibited a lower neutrophil apoptosis and clearance rates rather than an impaired neutrophil recruitment. Of note, the observation that heterozygous Sept4/ARTS^{+/-} mice display a partially aberrant phenotype implies a haploinsufficiency of ARTS in completing neutrophil apoptosis. As expected, the fraction of peritoneal macrophages increased with time (Fig. 1c). While at 24 h post induction we observed no difference between genotypes, at 66 h post induction, Sept4/ARTS^{-/-} mice displayed a significantly smaller fraction of

macrophages in comparison to WT mice (Fig. 1c). Notably, the eosinophil fraction remained unaffected (Fig. 1d). Female Sept4/ARTS^{-/-} mice displayed a larger fraction of neutrophils as well, together with smaller fractions of macrophages and eosinophils (Fig. S3a–c). Next, we further characterized the monocyte/macrophage sub-populations. Ly6C expression on monocytes/macrophages is associated with an inflammatory phenotype [67]. In line, and unlike unchallenged mice (Fig. S2h), we detected an increased Ly6C expression and a larger fraction of peritoneal Ly6C^{hi} monocyte/macrophages in ARTS^{-/-} mice (Fig. 1e–f). Macrophage reprogramming during resolution of inflammation is also associated with a reduction in F4/80 and CD11b surface expression [48]. Indeed, we observed a higher expression of F4/80 and CD11b in peritoneal macrophages of Sept4/ARTS^{-/-} mice in comparison to their WT counterparts (Fig. 1g–j). In addition, Sept4/ARTS^{-/-} mice displayed a larger fraction of CD11b^{high} macrophages and smaller fraction of CD11b^{low} macrophages as compared to WT mice (Fig. 1h–i). The CD11b^{high} subset of both Sept4/ARTS^{+/-} mice and Sept4/ARTS^{-/-} mice demonstrated an increased CD11b expression (Fig. 1–j). These observations suggest a hampered reprogramming of resolution phase macrophages to the pro-resolving F4/80^{low}CD11b^{low} phenotype [48]. In contrast, female Sept4/ARTS^{-/-} mice displayed a rather different phenotype: a reduced F4/80 expression (Fig. S3d) and no difference in CD11b subsets or expression (Fig. S3e–g). These findings suggest that hampered macrophage reprogramming during peritonitis is more evident in males than females. Hence, we decided to continue our analysis only with Sept4/ARTS^{-/-} males. Together, Sept4/ARTS^{-/-} mice retained higher numbers of peritoneal neutrophils at the expense of macrophages, which also failed to acquire a pro-resolving phenotype.

Peritoneal Sept4/ARTS^{-/-} leukocytes are apoptosis-resistant

The higher numbers of peritoneal neutrophils in Sept4/ARTS^{-/-} mice imply a failure in the execution of apoptosis in these cells during peritonitis. Not only neutrophils, but also macrophages undergo some degree of apoptosis during the resolution of inflammation [68]. Bearing in mind that all cell types in Sept4/ARTS^{-/-} mice are ARTS-deficient, we examined whether their respective leukocytes are apoptosis-resistant. BM-resident macrophages of unchallenged Sept4/ARTS^{-/-} mice expressed lower levels of cleaved PARP and caspase-9 (Fig. S4a), supporting diminished apoptosis in these cells. Using our peritonitis model, we compared the apoptotic properties of ARTS^{-/-} macrophages, macrophage-depleted leukocytes (MDLs, enriched with PMNs) and neutrophils (incubated ex vivo) to their WT counterparts. Indeed, we observed reduced expression of active

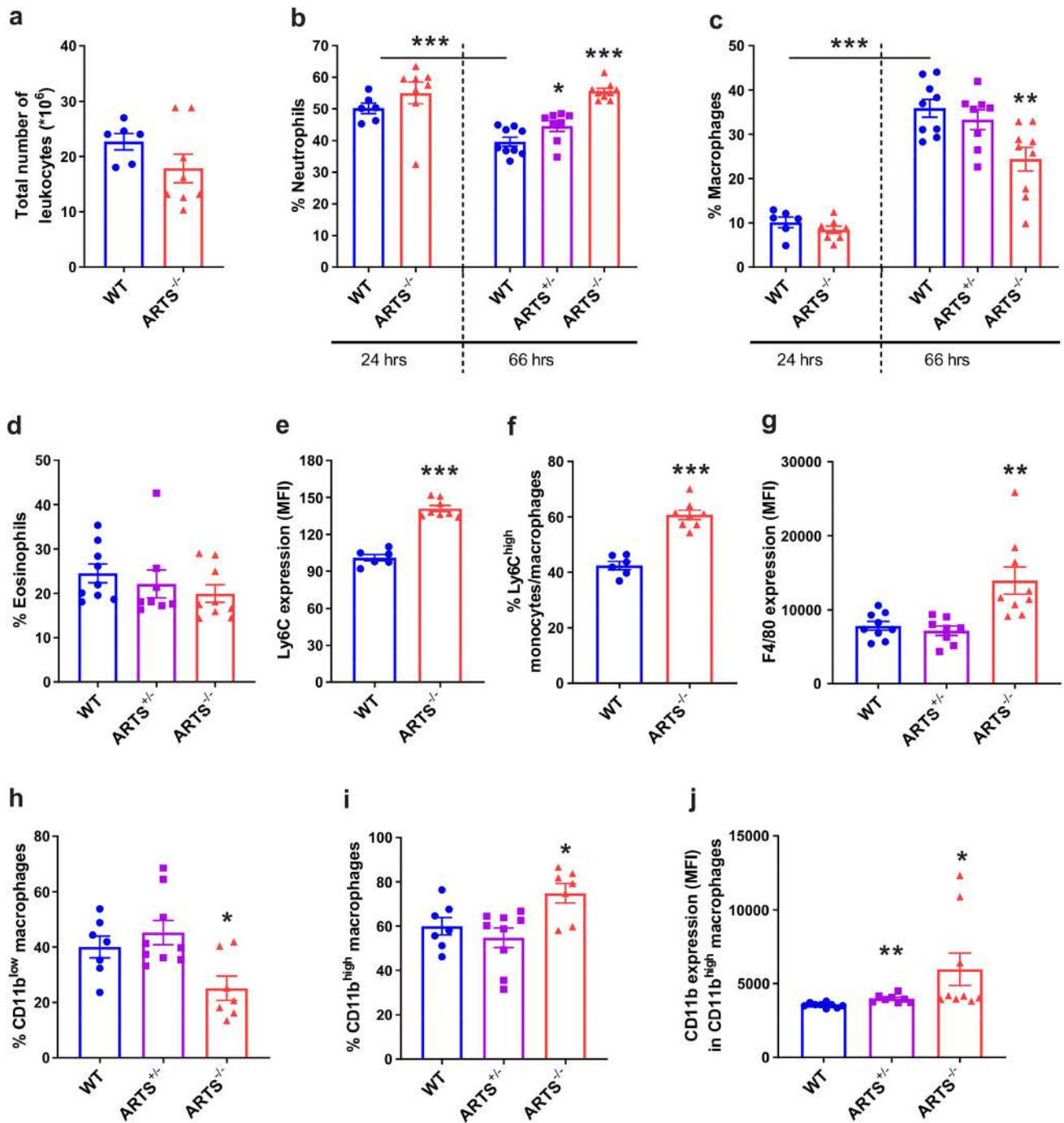


Fig. 1 Sept4/ARTS^{-/-} mice display an aberrant distribution of peritoneal leukocyte populations and macrophage reprogramming. Peritoneal exudate cells from male Sept4/ARTS^{+/+} (WT), Sept4/ARTS^{+/-} (ARTS^{+/-}) or Sept4/ARTS^{-/-} (ARTS^{-/-}) mice were collected 24 h or 66 h post zymosan A injection (1 mg/mouse). **a** Total number of peritoneal cells were counted 24 h post induction, n=6–8. **b–j** The cells were immunostained for leukocyte markers F4/80, Ly6C, Gr-1 (or Ly6G) and CD11b and analyzed by flow cytometry. **a–c** The average percentages of three leukocyte populations: F4/80⁺GR-1⁺(Ly6G⁺) neutrophils (**a**), F4/80⁺GR-1⁻CD11b⁺ macrophages (**b**) and F4/80⁺SSC^{hi} eosinophils (**c**), n=6–9. **d, e** The average mean

fluorescence intensity (MFI) of Ly6C expression on monocytes/macrophages (**d**) and the average percentage of Ly6C^{hi} monocytes/macrophages (**e**), n=6. (**f**) The average MFI of F4/80 expression on macrophages, n=8–9. **g–h** Macrophage sub-types were further gated according to CD11b expression, n=7–9. **i** The average MFI of CD11b expression within the CD11b^{high} macrophage subset, n=8–9. Altogether, the data summarize 2–3 independent experiments and are presented as mean ± SEM. Analysis by two-tailed Student's *t*-test; statistical significance is relative to the respective data from WT mice, * p < 0.05, ** p < 0.01, *** p < 0.001. Analysis by one-way ANOVA was statistically significant for all the results, except for (**c**)

(cleaved) caspase-9, H2AX/phospho-H2AX and full-length/cleaved PARP, all of which are classical apoptotic markers (Fig. 2a–c). Caspase-9 is the initiator in the caspase-dependent pathway [69], whereas H2AX (a histone) and PARP (a polymerase) are markers for DNA damage [70]. ARTS-deficient MDLs expressed reduced levels of active caspase-9 and PARP/cPARP at 66 h post peritonitis induction, whereas Sept4/ARTS^{-/-} neutrophils expressed reduced levels of these proteins after incubation *ex vivo* for 4 or 24 h (Fig. 2b, c). Furthermore, once peritoneal Sept4/ARTS^{-/-} macrophages and neutrophils were isolated and cultured for 24–48 h, they demonstrated an increased survival as compared to their WT counterparts (Fig. 2d, e). Notably, increased survival of these cells was not observed immediately after isolation (Fig. 2d, e). Of note, both cultured peritoneal Sept4/ARTS^{-/-} macrophages and neutrophils showed a delay in late apoptosis rate, whereas only Sept4/ARTS^{-/-} neutrophils showed a delay in early apoptosis rate (Fig. S4b–e). These findings indicate that ARTS^{-/-} macrophages and neutrophils retained their apoptotic-resistance *ex vivo*.

Sept4/ARTS^{-/-} macrophages display a hyperinflammatory phenotype with loss of pro-resolving properties

An increase in CD11b^{high} macrophages at the expense of CD11b^{low} macrophages, as observed in Sept4/ARTS^{-/-} mice undergoing peritonitis, may signify a shift towards a hyperinflammatory phenotype [48]. Therefore, we examined the inflammatory properties of isolated peritoneal macrophages. Compared to WT macrophages, Sept4/ARTS^{-/-} and Sept4/ARTS^{+/-} macrophages expressed increased amounts of iNOS, COX2 and MMP-9 (Fig. 2a), all of which are enzymes that characterize ongoing inflammation [43, 71, 72]. Strikingly, Sept4/ARTS^{-/-} macrophages also expressed reduced amounts of the pro-resolving mediators galectin-1 and 12/15-LO (Fig. 2a, f–g) [46, 66, 73]. ARTS^{-/-} macrophages also expressed lower CCR7 (Fig. 2a), which is essential in the chemoattraction of M1 and M2 macrophages [74]. Arginase-1, which is normally expressed in anti-inflammatory macrophages, but diminishes in the pro-resolving CD11b^{low} phenotype [45, 48], was increased in Sept4/ARTS^{-/-} macrophages (Fig. 2f–g). Together, these results underscore a hampered reprogramming to pro-resolving CD11b^{low} macrophages. Likewise, compared to WT MDLs, Sept4/ARTS^{-/-} MDLs expressed reduced amounts of pro-resolving effectors, including SphK, and chemokine receptors with scavenging capacity, like CCR5 and the silent receptor D6 (Fig. 2c) [57–59, 62]. The regulation of these scavenging chemokine receptors was specific as CCR2, an inflammatory chemokine receptor, was upregulated on Sept4/ARTS^{+/-} and Sept4/ARTS^{-/-} MDLs (Fig. 2b). In support of a blockage in acquiring a pro-resolving phenotype,

we detected reduced concentrations of TGF- β in the peritoneal exudates of ARTS^{+/-} and ARTS^{-/-} mice as compared to WT mice (Fig. 2h). TGF- β is a key cytokine upregulated during the resolution of peritonitis and an essential mediator of resolution of inflammation [75, 76]. Of note, the reduction in TGF- β concentrations was more substantial in Sept4/ARTS^{-/-} mice as compared to Sept4/ARTS^{+/-} mice (Fig. 2h). Efferocytosis is a key step in resolution of inflammation [40–42, 55]. Measurement of apoptotic cell efferocytosis can be done by counting the number of engulfed cells per macrophage. To determine whether ARTS regulates efferocytosis during the resolution of peritonitis, we isolated WT and Sept4/ARTS^{-/-} macrophages 66 h post induction. We then measured apoptotic cell uptake by counting the number of engulfed cells (i.e., mouse PMNs) per macrophage. In this *in vivo* analysis, we revealed that Sept4/ARTS^{-/-} mice exhibited impaired efferocytosis as indicated by a lower average number of engulfed cells per macrophage, a lower percentage of phagocytic macrophages, and a lower phagocytic efficiency index (Fig. 3a–d). To confirm a reduced phagocytic efficiency of Sept4/ARTS^{-/-} macrophages in comparison to WT macrophages, we isolated macrophages at an earlier time point of 48 h post induction and incubated them with pre-labelled apoptotic Jurkat T cells. In this *ex vivo* assay, we confirmed that Sept4/ARTS^{-/-} macrophages had an impaired ability of apoptotic cell uptake as indicated by a lower average number of apoptotic cells per macrophage, a lower percentage of phagocytic macrophages, and a lower phagocytic efficiency index (Fig. 3e–h). Eosinophils play a role in regulating inflammation and its resolution by storing preformed cytokines, chemokines and growth factors, available for immediate release [50, 51]. Peritoneal Sept4/ARTS^{-/-} eosinophils demonstrated higher granularity, analyzed by flow cytometry, when compared to their WT counterparts (Fig. S4f). Thus, eosinophil degranulation seems to be hampered in ARTS^{-/-} mice. Considering the pro-resolving role of eosinophils in peritonitis [50, 51], these findings are in accord with the hyperinflammatory state of these mice. Altogether, based on our peritonitis model, Sept4/ARTS^{-/-} macrophages manifested a hyperinflammatory phenotype, which was associated with loss of pro-resolving properties and an impaired efferocytosis.

Peritoneal Sept4/ARTS^{-/-} macrophages display a pro-inflammatory cytokine repertoire

Macrophage reprogramming is accompanied by reduced secretion of pro-inflammatory cytokines and increased secretion of anti-inflammatory cytokines upon exposure to bacterial stimuli; this can be measured *ex vivo* by exposure of macrophages to LPS [48]. We applied this methodology first to isolated BM-, spleen- and peritoneal-resident macrophages of unchallenged WT and Sept4/ARTS^{-/-} mice. While LPS

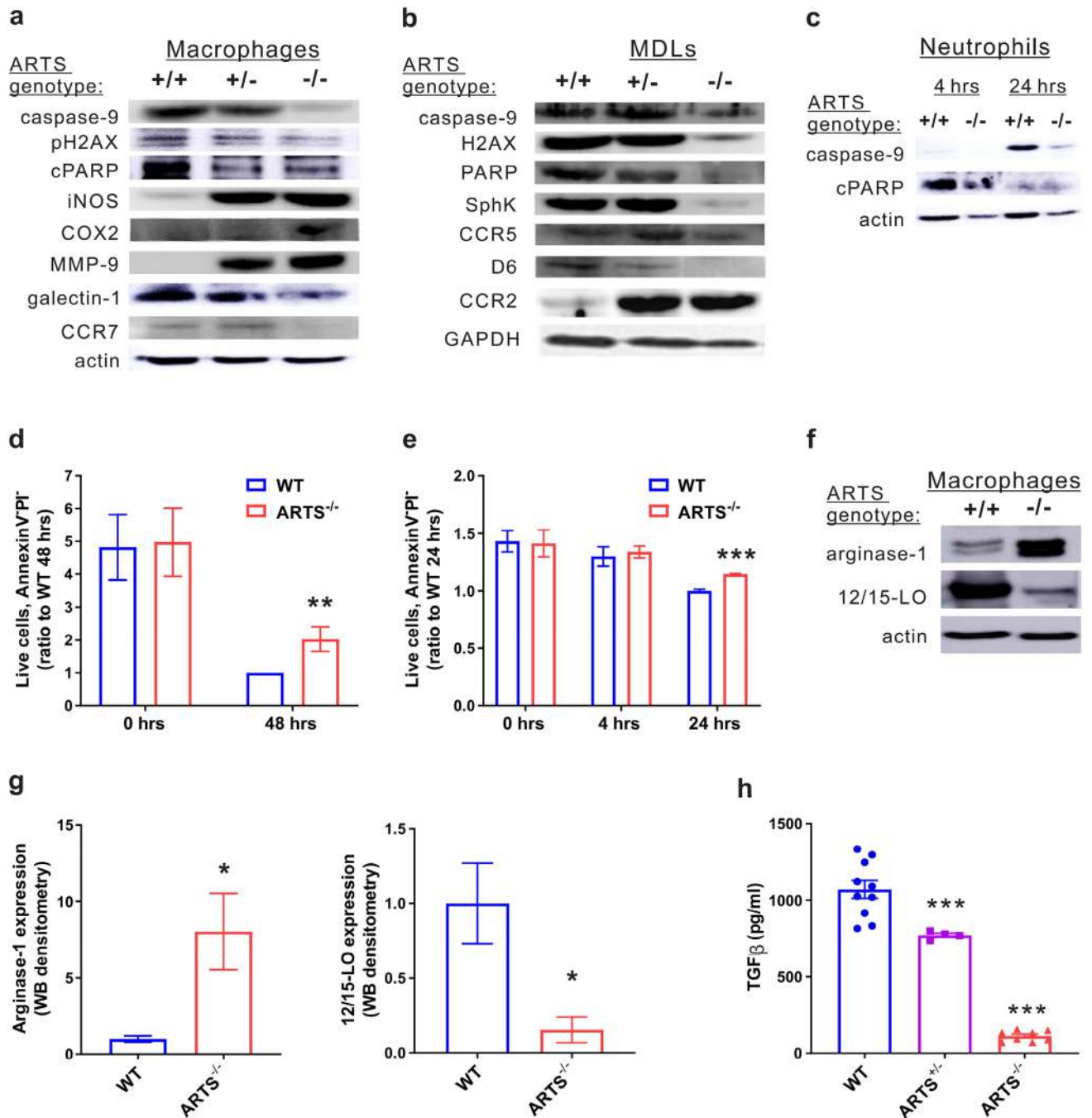


Fig. 2 Peritoneal Sept4/ARTS^{-/-} leukocytes are apoptosis-resistant and display a hyperinflammatory phenotype with loss of pro-resolving properties. Peritoneal exudate cells from male Sept4/ARTS^{+/+} (WT), Sept4/ARTS^{+/-} (ARTS^{+/-}) or Sept4/ARTS^{-/-} (ARTS^{-/-}) mice were collected from unchallenged mice or 4, 24 or 66 h post zymosan A injection (1 mg/mouse). **a–c**, **f–g** Peritoneal F4/80⁺ macrophages were isolated and separated from F4/80⁻ leukocytes (*i.e.* macrophage-depleted cells, MDLs). Cell pellets were lysed, run by SDS-PAGE and blotted with antibodies against the indicated proteins, with GAPDH or actin serving as controls. The membranes were then reacted with matching HRP-conjugated secondary antibodies and developed. **a–c**, **f** Representative blot images. **g** Densitometry analysis. **d** Peritoneal F4/80⁺ macrophages were isolated 66 h post zymosan A injection. Then, they were cultured or not for

48 h, followed by staining with Annexin V and PI and analysis by flow cytometry, $n=4$. **c–d** Peritoneal Ly6G⁺ neutrophils were isolated 24 h post zymosan A injection. Then, they were cultured for 4 or 24 h, followed by staining with Annexin V and PI and analysis by flow cytometry, $n=3–4$. **d**, **e** Peritoneal macrophages or neutrophils were cultured for 0, 4, 24 or 48 h; followed by staining with Annexin V and PI and analysis by flow cytometry, $n=3–4$. **h** Cell-free peritoneal exudates were analyzed for TGF- β levels using a standard ELISA. Analysis by one-way ANOVA confirmed statistical significance. Altogether, the data summarize 2–4 experiments and are presented as mean \pm SEM. Analysis by two-tailed Student's *t*-test; statistical significance is relative to the respective data from WT mice, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

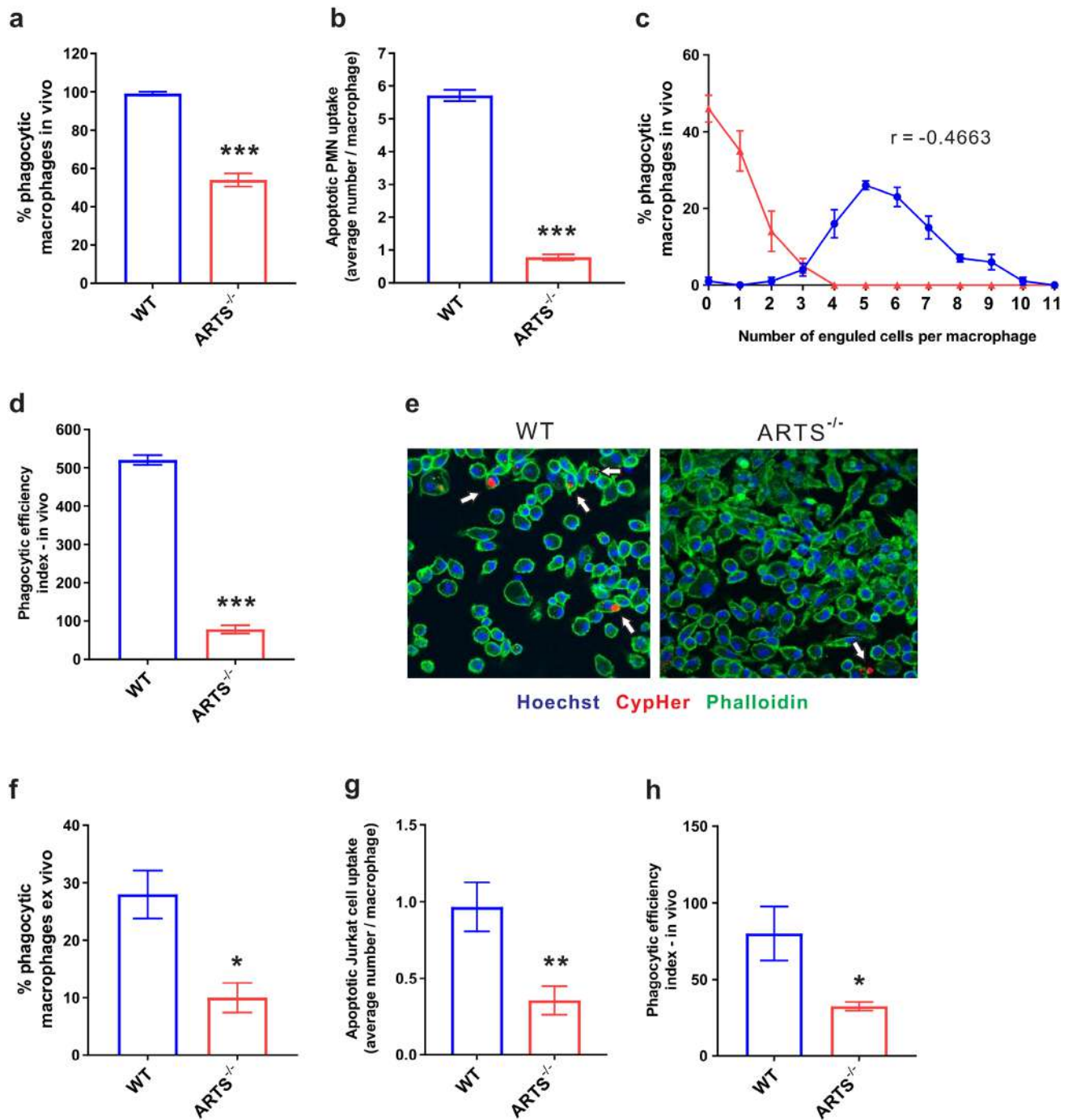
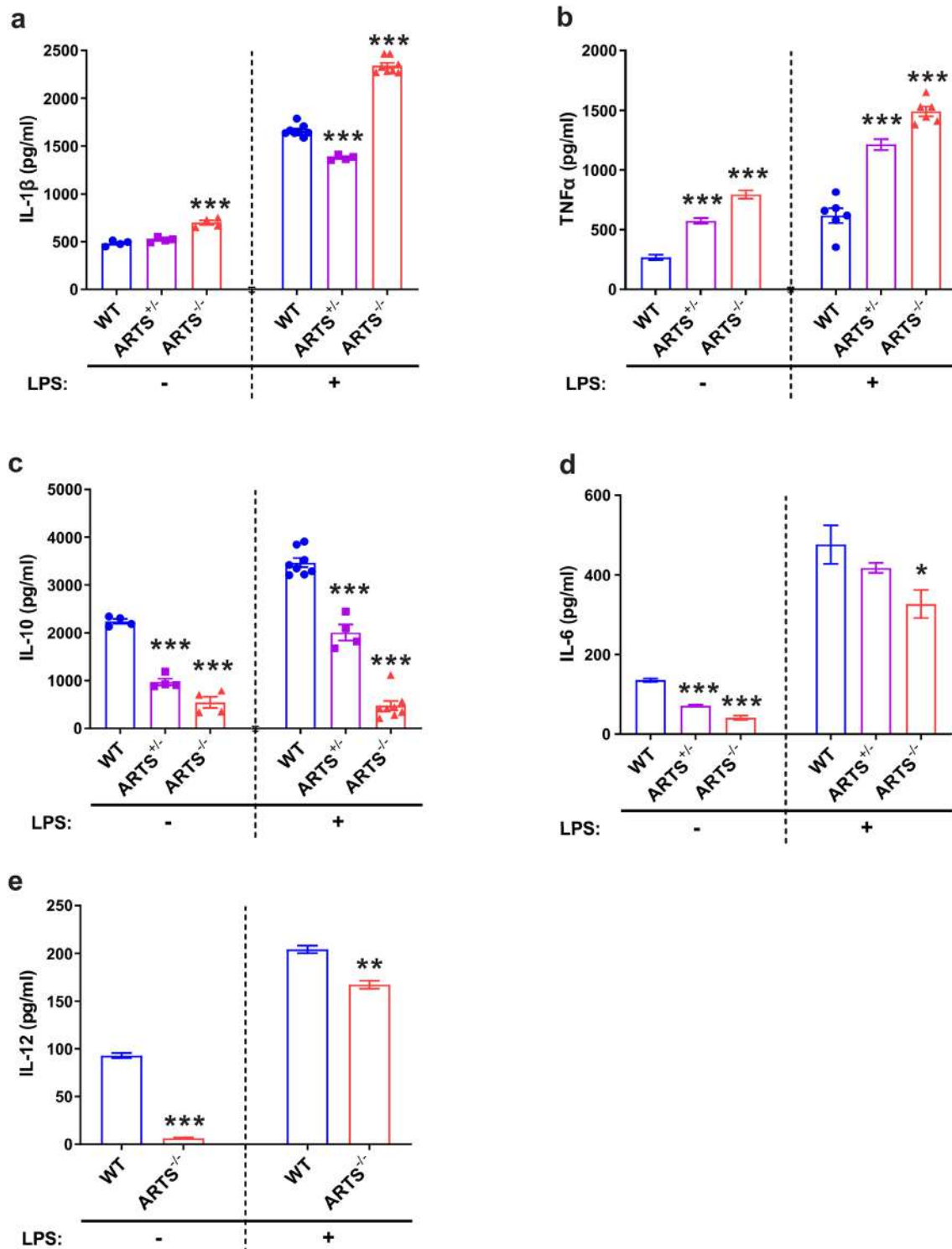


Fig. 3 Peritoneal Sept4/ARTS^{-/-} macrophages exert impaired efferocytosis. Peritoneal F4/80⁺ macrophages were isolated from male Sept4/ARTS^{+/+} (WT) or Sept4/ARTS^{-/-} (ARTS^{-/-}) mice 66 h (a–d) or 48 h (e–h) post zymosan A injection (1 mg/mouse). The isolated cells were stained with Hoechst for nuclear detection and phalloidin-FITC for F-Actin. **a–d** The macrophages were enumerated for apoptotic PMN uptake in vivo (nuclei in macrophage cytoplasm). **e–h** Jurkat T cells underwent staurosporine-induced apoptosis, stained with CypHer5E Mono NHS Ester, after which they were incubated with isolated macrophages for 4 h. The macrophages were enumerated for apoptotic cell uptake ex vivo (red-labelled phagocytosed cells). The percentage of phagocytic macrophages engulfing discrete numbers

of apoptotic cells (**a, c, f**) and the average number of apoptotic cells engulfed per macrophage (**b, g**) were calculated. **e** Representative images. Arrows point at labelled engulfed cells. (**d, h**) The phagocytic efficiency index was calculated as previously described [63]. The data is averaged from 4 coverslips generated from 2–3 mice in 2 separate experiments – in each slide at least 50 macrophages were analyzed. The data are presented as mean \pm SEM. Analysis by two-tailed Student's *t*-test; statistical significance is relative to the respective data from WT mice, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. In **c**, the Pearson analysis shows no correlation between WT and ARTS^{-/-} macrophages, suggesting a statistically different engulfment capacity



increased cytokine expression regardless of genotype and source of cells, there were notable difference in LPS-induced cytokine secretion between Sept4/ARTS^{-/-} and WT macrophages (Fig. S5). Spleen-derived Sept4/ARTS^{-/-} macrophages demonstrated reduced LPS-induced secretion of pro- and anti-inflammatory cytokines (Fig. S5a–c), whereas

BM-resident Sept4/ARTS^{-/-} macrophages secreted similar levels of all cytokines as compared with WT macrophages (Fig. S5d–f). On the contrary, in response to LPS, resident peritoneal Sept4/ARTS^{-/-} macrophages secreted higher levels of pro-inflammatory TNF- α and IL-6 and lower levels of the anti-inflammatory cytokine IL-10 (Fig. S5g–i), suggesting

Fig. 4 Peritoneal Sept4/ARTS^{-/-} macrophages display a pro-inflammatory cytokine repertoire. Peritoneal F4/80⁺ macrophages were isolated from male Sept4/ARTS^{+/+} (WT), Sept4/ARTS^{+/-} (ARTS^{+/-}) or Sept4/ARTS^{-/-} (ARTS^{-/-}) mice 66 h post zymosan A injection (1 mg/mouse). Then, the macrophages were cultured and activated or not with LPS (1 µg/ml) for 24 h. The cell-free supernatants were collected and analyzed for an array of cytokines, using a standard ELISA. **a** IL-1β, n=4–8. **b** TNF-α, n=5–9. **c** IL-10, n=4–8. **d** IL-6, n=3. **e** IL-12, n=3. Altogether, the data summarize 3 experiments, including at least 2 technical replicates, and are presented as mean ± SEM. Analysis by two-tailed Student's *t*-test; statistical significance is relative to the respective data from WT mice (either with or without LPS, bordered by a dashed line), * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001. Additionally, the data were statistically significant in all groups when cytokine levels were compared between LPS treatment and no treatment, except for IL-10 levels in the ARTS^{-/-} macrophage group. Analysis by two-way ANOVA was statistically significant for all the results, except for (d). **d** An interaction between the bars with and without LPS could not be excluded. However, one-way ANOVA was statistically significant both ways in all figures

a hyper-inflammatory phenotype. Notably, the differences in cytokine secretion between WT and Sept4/ARTS^{-/-} resident peritoneal macrophages were evident only in LPS-stimulated peritoneal macrophages and not in vehicle-treated ones. Next, we stimulated resolution phase peritoneal macrophages obtained from WT and Sept4/ARTS^{-/-} mice with LPS. Our results indicated a shift towards an inflammatory cytokine repertoire once Sept4/ARTS^{-/-} macrophages were compared to WT controls, regardless of LPS treatment (Fig. 4). Sept4/ARTS^{-/-} macrophages also secreted higher levels of the pro-inflammatory cytokines IL-1β and TNF-α as compared to WT controls (Fig. 4a, b), whereas Sept4/ARTS^{+/-} macrophages secreted higher levels of TNF-α, but not IL-1β (Fig. 4a, b). Conversely, ARTS^{+/-} and ARTS^{-/-} macrophages secreted lower levels of the anti-inflammatory cytokine IL-10 as compared to controls (Fig. 4c). Surprisingly, resolution phase ARTS^{-/-} macrophages secreted lower levels of the cytokines IL-6 and IL-12 in comparison to WT controls (Fig. 4d, e), although these cytokines are considered pro-inflammatory in nature [77, 78]. As expected, LPS per se significantly increased the secretion of IL-1β, TNF-α, IL-6 and IL-12, regardless of macrophage genotype (Fig. 4a, b, d, e). LPS also increased the secretion of IL-10 from WT and ARTS^{+/-} macrophages but was ineffective in Sept4/ARTS^{-/-} macrophages (Fig. 4c). In summary, resolution phase Sept4/ARTS^{-/-} macrophages exhibited a hyperinflammatory phenotype in vivo, consistent with hampered reprogramming by apoptotic PMN uptake, which was mostly retained ex vivo following macrophage isolation.

Overexpression of ARTS in lymphocytes intensifies their immune-silencing properties upon apoptosis

To mimic the process of efferocytosis in vivo and obtain a more direct view of the role played by ARTS in macrophage

reprogramming, we used a co-culture model. Co-culturing LPS-activated macrophages with apoptotic leukocytes results in suppression of their inflammatory properties, which among others include reduction in the secretion of pro-inflammatory cytokines [48, 79]. Based on this experimental rationale, we induced apoptosis in human Jurkat cells, using UV irradiation or an incubation with STS, followed by co-culturing with LPS-activated human THP-1 macrophages. As expected, co-culture of macrophages with UV-irradiated or STS-treated Jurkat cells resulted in reduced IL-1β and TNF-α secretion, when compared to LPS-treated macrophages alone or to a co-culture with live Jurkat cells (Fig. 5a, b). Taking into account that ARTS overexpression induces apoptosis [27, 64], we transfected human Jurkat cells with a vector overexpressing ARTS or with a control vector. The transfected cells were co-cultured with LPS-activated human THP-1 macrophages. In a similar manner to the control experiments, co-culture of macrophages with ARTS-transfected Jurkat cells effectively reduced the secretion of IL-1β as compared to control transfected cells (Fig. 5a). We also observed a reduction in TNF-α secretion, but only in higher ratios of apoptotic cells to macrophages (Fig. 5b). In addition to the ability to suppress secretion of pro-inflammatory cytokines, we determined whether ARTS-transfected lymphocytes also increased the expression of pro-resolving effectors, in comparison to Sept4/ARTS^{-/-} leukocytes. We previously reported that CCR5⁺ apoptotic leukocytes scavenge chemokines that usually attract macrophages to inflamed sites in a peritonitis mouse model [59]. First, we validated that Jurkat cells did increase CCR5 expression following induction of apoptosis by UV irradiation (Fig. 5c). Next, we examined CCR5 expression in ARTS-transfected Jurkat cells as compared to control transfected Jurkat cells. Indeed, ARTS overexpression was sufficient to induce high expression of CCR5 (Fig. 5d). Taken together, the outcome of ARTS overexpression in vitro is consistent with our observations in Sept4/ARTS^{-/-} mice, showing that ARTS expression in apoptotic leukocytes is involved in the cross-talk with macrophages during the resolution of inflammation.

Discussion

Acute inflammation due to bacterial infection or injury is normally characterized by the rapid influx of blood PMNs, typically neutrophils, followed swiftly by recruited monocytes that mature into inflammatory macrophages [40, 41]. Resolution of inflammation can occur only if PMNs are efficiently eliminated by efferocytosis, avoiding local tissue reaction, and the mononuclear cells (macrophages, dendritic cells and lymphocytes) depart, returning to homeostatic numbers and phenotypes [40–44]. Chronic diseases, such

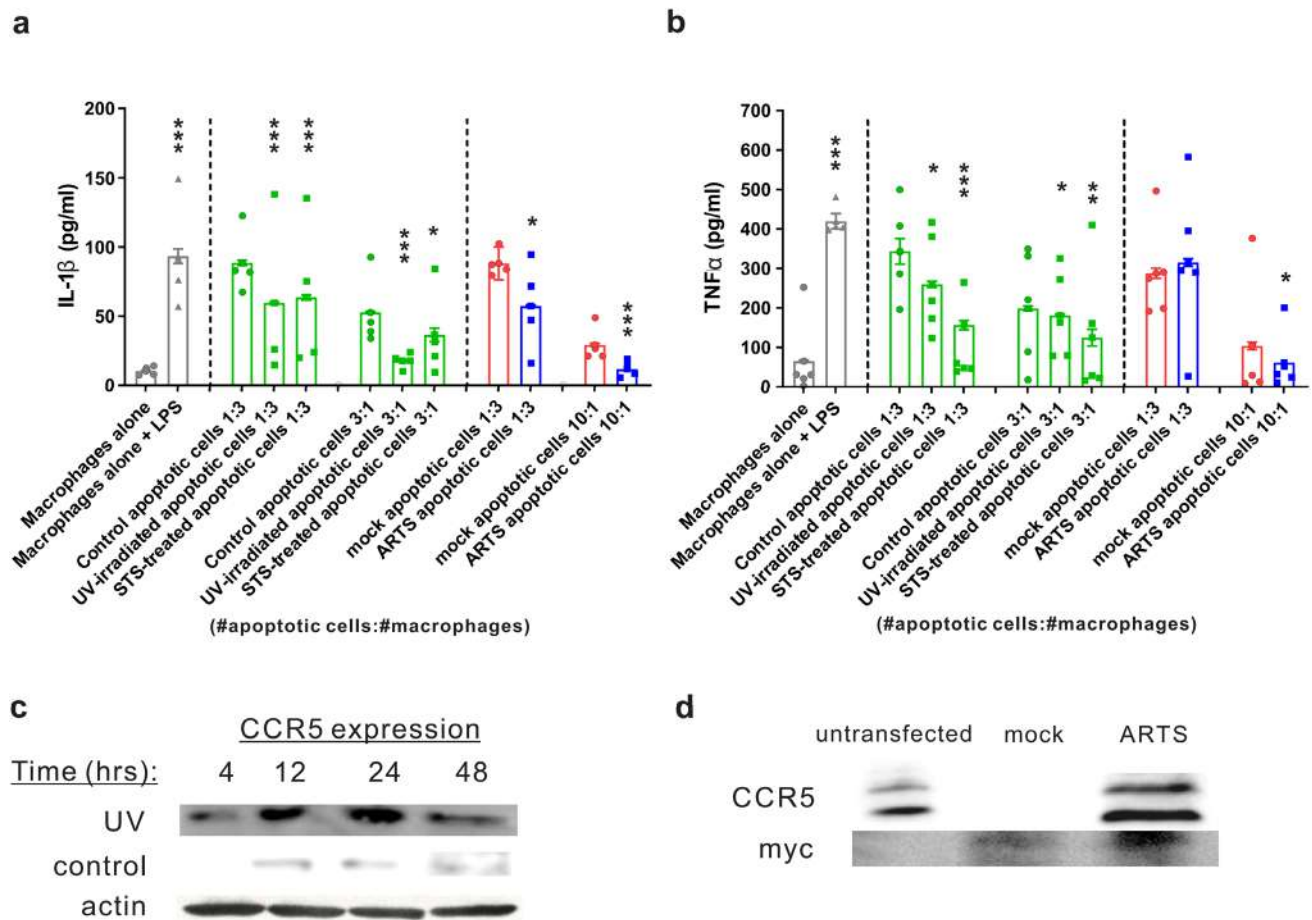


Fig. 5 Overexpression of ARTS in leukocytes promotes their immune-silencing properties upon apoptosis. Human Jurkat cells were either exposed to UV irradiation (5 min) or treated with STS (1 μ M) to induce apoptosis. Alternatively, the cells were transfected with plasmids encoding c-Myc-tagged ARTS or a control c-Myc vector. **a, b** After 24 h, live or apoptotic Jurkat cells were co-cultured with THP-1-derived macrophages at the indicated ratios. Next, the co-cultured cells were activated with LPS (500 ng/ml) for additional 24 h. Finally, the cell-free supernatants were collected and analyzed for the content of the cytokines IL-1 β (**a**) and TNF- α (**b**), using a standard ELISA. Altogether, the data summarizes 4–5 experiments with 3–4 replicates per experiment. The data are presented as mean

per experiment \pm SEM of all replicates. Analysis by two-tailed Student's *t*-test; statistical significance is relative to the respective data from matched-control or mock cells, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Analysis by one-way ANOVA confirmed statistical significance. **c, d** UV-irradiated or vector-transfected human Jurkat cells were lysed. The lysates were run by SDS-PAGE and blotted with antibodies against human CCR5, actin (loading control) or c-Myc (transfection control). The membranes were then reacted with matching HRP-conjugated secondary antibodies and developed. Results are representative blot images. **c** CCR5 expression following UV irradiation at the indicated time points. **d** CCR5 expression in transfected and untransfected cells

as rheumatoid arthritis, diabetes, atherosclerosis, asthma and colitis, are often associated with a hyper-inflammatory phenotype that fails to resolve [54, 80–85]. Thus, the resolution phase of inflammatory episodes has to be highly coordinated and regulated, involving distinct immunomodulatory mechanisms, among which macrophage reprogramming and the cross-talk between pro-resolving macrophages and engulfed apoptotic PMNs are key [40, 41]. We and others have shown that apoptotic cells sequester inflammatory chemokines [59, 60], release immunomodulatory factors [46, 47, 57, 66, 86], and that the mere uptake of apoptotic cells suppresses release of pro-inflammatory cytokines from macrophages and promotes the secretion of anti-inflammatory cytokines

[43, 48, 79]. It is therefore reasonable to hypothesize that impinging the efferocytic process in macrophages would result in halted reprogramming and a sustained inflammatory condition.

To examine this hypothesis, we utilized a model of spontaneously-resolving peritonitis in apoptosis-deficient Sept4/ARTS^{-/-} mice, characterized with resistance to cell death [36–38]. In steady state conditions, we observed a higher number of neutrophils in the BM of male Sept4/ARTS^{-/-} mice, but not in the peritoneum or the spleen. During the early inflammatory phase (24 h) of peritonitis, similar peritoneal neutrophil and macrophage numbers were observed in WT and Sept4/ARTS^{-/-} mice, although

the neutrophils showed partial apoptosis resistance in these mice. On the other hand, at 66 h, peritoneal neutrophils demonstrated higher numbers, increased survival and reduced apoptosis *in vivo*. These results lead us to reason that peritoneal Sept4/ARTS^{-/-} neutrophils failed to carry out effective apoptosis, and consequently were not efferocytosed by resolution phase macrophages resulting in diminished engulfment-related signaling, impaired reprogramming and sustained inflammation. Indeed, macrophages isolated from Sept4/ARTS^{-/-} mice retained their inflammatory phenotype, associated with higher F4/80, CD11b, Ly6C, iNOS, COX2 and MMP-9 expression. Moreover, these hyperinflammatory macrophages secreted higher levels of the pro-inflammatory cytokines IL-1 β and TNF- α and lower levels of the anti-inflammatory cytokines TGF- β and IL-10. A similar response was observed in LPS-treated resident peritoneal, but not BM or splenic macrophages. In this model of zymosan A peritonitis, resident peritoneal macrophages emigrate to remote organs and BM-originating monocyte-derived macrophages constitute most of the resolution-phase macrophage population [48, 87]. Therefore, it is unlikely that sustained presence of resident peritoneal macrophages drives the hyper-inflammatory phenotype of Sept4/ARTS^{-/-} mice; it is rather the defective reprogramming of Sept4/ARTS^{-/-} macrophages. Moreover, Sept4/ARTS^{-/-} macrophages did not increase in numbers, favoring a reprogramming blockage over the possibility of enhanced inflammatory response via increased macrophage recruitment or proliferation. In line with the hyper-inflammatory phenotype, Sept4/ARTS^{-/-} macrophages demonstrated an impaired capacity to uptake PMNs *in vivo* or apoptotic cells *ex vivo*, which probably leads to halted reprogramming. Notably, ARTS^{-/-} mice did not display a classical enhanced inflammation, since the secretion of pro-inflammatory cytokines, such as IL-6 and IL-12, were unexpectedly reduced from their macrophages. Moreover, our RNA-Seq analysis indicates that satiated resolution-phase macrophages, which are generated by self-limiting efferocytosis, express higher levels of IL-6 and IL-12 in comparison to their phagocytic counterparts [87]. Therefore, blocked reprogramming may reduce the levels of these cytokines. Notably, a similar macrophage phenotype was observed in D6/ACKR2-deficient macrophages during the resolution of peritonitis [58]. These macrophages exhibited defective reprogramming due to hampered signaling from apoptotic PMN, resulting in hampered resolution of inflammation [58, 62]. Of note, macrophages isolated from female Sept4/ARTS^{-/-} mice seemingly secreted higher levels of IL-12 as compared to WT females, while IL-6 levels remained intact (data not shown). Nonetheless, macrophages isolated from Sept4/ARTS^{-/-} females did not display an impaired reprogramming as observed in macrophages isolated from Sept4/

ARTS^{-/-} males. This observation is in line with the positive effect of estrogen on resolution of inflammation [88], supporting a more prominent peritonitis in Sept4/ARTS^{-/-} males as compared to Sept4/ARTS^{-/-} females.

In a direct cause and effect, these findings showed that inhibiting effective efferocytosis by modes of inhibiting apoptosis in PMNs blocks macrophage reprogramming. In support, the cyclin-dependent kinase inhibitor, R-roscovitin, induces neutrophil apoptosis and consequently enhances macrophage reprogramming and resolution of inflammation in various acute models [62, 89]. Moreover, TNF-related apoptosis-inducing ligand (TRAIL)-deficient mice also display higher neutrophil numbers due to apoptosis-resistance, consequently leading to an enhanced inflammatory response following an induction of peritonitis [90]. The authors claimed that TRAIL^{null} mice displayed a delayed neutrophil apoptosis together with a delayed resolution of inflammation [90]. We have yet to test whether in Sept4/ARTS^{-/-} mice delayed apoptosis and resolution of inflammation take place rather than their absolute blockage. There might be an unknown ARTS-independent pathway that can trigger a compensatory apoptosis in leukocytes; for example, the apoptosis rate of Sept4/ARTS^{-/-} lymphocytes was similar to their WT counterparts [36]. Still, at a time point of 66 h post-induction, when normally resolution takes place [48, 65], we observed reduced expression of pro-resolving effectors in macrophages isolated from Sept4/ARTS^{-/-} mice, including TGF- β , 12/15-LO, galectin-1 and CCR7. In our study, eosinophils, which also participate in the resolution of inflammation [51], exhibited impaired degranulation. Follow-up studies will determine whether this aberrant phenotype of macrophages and eosinophils leads to reduced production of specialized pro-resolving lipid mediators. It should be mentioned that effects that were evident in ARTS^{-/-} mice were also evident to some extent in ARTS^{+/-} mice, suggesting haploinsufficiency of ARTS signaling in mediating apoptosis and resolution of inflammation.

One may suggest that ARTS deficiency in macrophages per se halted their reprogramming rather than ARTS-mediated apoptosis in PMNs. We examined this possibility by performing co-culture experiments. Incubating ARTS-overexpressing Jurkat cells together with macrophages resulted in inhibited secretion of pro-inflammatory IL-1 β and TNF- α as effectively as incubation with Jurkat cells that underwent classically-induced apoptosis. ARTS-overexpressing Jurkat cells also increased CCR5 expression in a similar manner to classically-induced apoptotic Jurkat cells. These results are consistent with a cell-autonomous mechanism induced by ARTS in PMNs; ARTS deficiency in apoptotic PMNs rather than in macrophages seems to be the mechanism by which macrophages retain their inflammatory properties. In line with these results, Sept4/ARTS^{-/-} PMNs expressed reduced levels of pro-resolving receptors, such as CCR5 and

D6, which sequester inflammatory chemokines [59–61], and SphK, which synthesizes the anti-inflammatory lipid S1P [56, 57]. Further mechanistic insight is required to determine how ARTS-mediated apoptosis develops in both types of leukocytes. Our findings are of clinical importance, since therapeutic induction of apoptosis in leukocytes, including ARTS activation [91], may facilitate resolution of undesired harmful inflammation.

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Author contributions NM, ZZZ, PK, OZT, and SSZ performed experiments and analyzed the results. SSZ assisted in planning the experiments. SL and AA planned the experiments, analyzed the data and wrote the manuscript.

Compliance with ethical standards

Competing interests: The authors declare no competing interests.

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