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Immune serum–activated human macrophages coordinate with eosinophils to immobilize *Ascaris suum* larvae

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Abstract

Helminth infection represents a major health problem causing approximately 5 million disability-adjusted life years worldwide. Concerns that repeated anti-helminthic treatment may lead to drug resistance render it important that vaccines are developed but will require increased understanding of the immune-mediated cellular and antibody responses to helminth infection. IL-4 or antibody-activated murine macrophages are known to immobilize parasitic nematode larvae, but few studies have addressed whether this is translatable to human macrophages. In the current study, we investigated the capacity of human macrophages to recognize and attack larval stages of *Ascaris suum*, a natural porcine parasite that is genetically similar to the human helminth *Ascaris lumbricoides*. Human macrophages were able to adhere to and trap *A suum* larvae in the presence of either human or pig serum containing *Ascaris*-specific antibodies and other factors. Gene expression analysis of serum-activated macrophages revealed that CCL24, a potent eosinophil attractant, was the most upregulated gene following culture with *A suum* larvae in vitro, and human eosinophils displayed even greater ability to adhere to, and trap, *A suum* larvae. These data suggest that immune serum–activated macrophages can recruit eosinophils to the site of infection, where they act in concert to immobilize tissue-migrating *Ascaris* larvae.

KEYWORDS

antibodies, Ascariasis, Eosinophils, Helminth, immune serum, Macrophage

1 | INTRODUCTION

More than 1.5 billion people worldwide are infected with one or more soil-transmitted helminths (STH) which include the nematodes roundworm *Ascaris lumbricoides*, the whipworm *Trichuris trichiura*, and the hookworms *Ancylostoma duodenale* and *Necator americanus*.[1,2] *Ascaris lumbricoides* accounts for approximately 0.8-1.2 billion of those infected with STH and more than 1 million disability-adjusted life years (DALYs).[3] Ascarisias contributes significantly to childhood mortality rates associated with STH infections due to heavy worm burdens and associated intestinal obstruction and/or hepatobiliary and pancreatic dysfunction.[4-6] Current treatment

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strategies in STH-endemic areas (including sub-Saharan Africa, the Americas, East Asia and China) include preventive chemotherapy (using anti-helminth drugs such as benzimidazoles), coupled with community-wide improvements in sanitation, water management and education. Although these interventions have improved rates of STH infection, repeated anti-helminth treatment in livestock has led to drug resistance, a phenomenon that is feared will occur in humans.[7] To date, no successful vaccine against *A lumbricoides* has been formulated, and although there is no substantiated data of anti-helminthic resistance in humans, the reduced efficacy of albendazole treatment was recently reported in small cohort of Rwandan schoolchildren.[8] The development of an effective vaccine relies on the identification of correlates of protection, whereby the induced immune response supports efficient trapping or killing of the larvae as they migrate through the tissues.

Further insight for the identification of correlates of protection could be provided by the study of humoral and cellular mechanisms that confer protection in animal models. *A suum* is the infectious agent of porcine ascariasis, which is closely related to the human helminth A lumbricoides. Both parasites show striking similarities in their morphology, biology and genetics with both exhibiting anthropozoonotic potential.[9-11] Accordingly, it has been suggested that the two parasites actually belong to the same species,[12] with *A suum* representing an excellent model to study the common human nematode. Human *Ascaris* infection elicits a highly polarized type 2 immune response, characterized by specific IL-4 and IL-5 cytokines, and peripheral eosinophilia due to larval migration within the lung space.[13-15] In pigs, the response to infection is better defined, whereby infected animals exhibit classical features of a type 2 immunity, including eosinophilia, mastocytosis, macrophage expansion, antibody production, enhanced intestinal motility and mucus secretion.[16,17] Studies from endemic regions have also indicated a potential protective role for antibodies, with the presence of specific Ig (immunoglobulin)-E antibodies to parasite antigens, such as ABA-1, facilitating protective immunity and correlating to lower rates of re-infection in humans.[18-20] Pigs can also generate strong protective immunity after a prolonged or repeated exposure to *Ascaris*,[21] which is mediated through resistance at the intestinal barrier during larval migration.[22] Protection can also be induced experimentally by immunization with egg, larval or adult *A suum* products, with resistant animals (both mice and pigs) exhibiting elevated antigen-specific antibody and cellular responses.[23-25] Lastly, characterization of *A suum* infection in mice demonstrated that a single low-dose infection induced protective immunity against a subsequent challenge, coinciding with elevated numbers of Ig-expressing cells at sites of larval migration.[26]

Type 2 immune cell infiltrates or granulomas are often found surrounding tissue-migrating helminth larvae of immune animals and are predominately composed of macrophages and eosinophils.[27] Animal models have shown that antibodies present in the immune serum of mice infected with the murine nematode *H polygyrus* can activate macrophages to adhere to the larvae in both tissue granulomas in vivo[28] and in macrophage-larvae co-cultures in vitro.[29,30] Murine macrophages can also be activated by IL-4 to adhere to and immobilize the rodent hookworm, *N brasiliensis* larvae both in vivo and in vitro.[28,31] These macrophages typically exhibit an alternatively activated (AAM) phenotype characterized by expression of Arginase-1 (Arg1), and Arg-1 was demonstrated to be essential for protective immunity against *N brasiliensis* in vivo[28,31] and immobilization of *H polygyrus* larvae in vitro.[29]

By contrast, eosinophils only play a minor role in protective immunity against *N brasiliensis*[32] and seem to contribute to the clearance of larval debris rather than larval killing as observed in *H polygyrus*-infected mice.[33] Nevertheless, antibody-activated eosinophils have been shown to kill nematode larvae in vivo for *Trichinella spiralis*-infected mice,[34] or in vitro for human eosinophils and *T spiralis* larvae,[35] and for ruminant eosinophils and *Haemonchus contortus*.[36] Eosinophils are also known to play a crucial role in immunity during *Ascaris* infection, where eosinophil-mediated protection from larval stages in the lungs occurs in mice following pre-sensitization with airway allergens[37] and in vitro, with antibody-activated porcine eosinophils shown to degranulate and kill third-stage (L3) larvae.[38]

Although the capacity of antibody-activated eosinophils to attack larvae has been studied across numerous host and parasite models, the ability of human macrophages to kill nematode larvae has yet to be investigated. The only exception to this is a report from Bonne-Annee *et al*, showing that human macrophages collaborate with neutrophils to kill *Strongyloides stercoralis* larvae.[39] Moreover, translation of the murine studies to humans is lacking and gene expression by murine and human alternative activated macrophages differs with human macrophages are typically described to lack Arg-1 expression.[40] In the current study, we sought to determine whether human macrophages can replicate the capacity of murine macrophages to recognize and immobilize parasitic nematodes. We found that human macrophages, activated by serum from *A suum*-infected pigs or *A lumbricoides*-infected patients, could adhere to and immobilize *A suum* larvae in vitro and that this occurred in an Arg-1–independent manner. Interestingly, RNA sequencing analysis revealed that CCL24, an eosinophil chemoattractant, was the most highly upregulated gene in the macrophages and immune serum plus *Ascaris*-activated human eosinophils was even more efficient than macrophages at adhering to and immobilizing larvae. Based on these data, we propose a mechanism by which components present in immune serum activate macrophages and eosinophils to co-operate in mediating anti-larval tissue responses following repeated infections with *Ascaris*.

2 | IMMUNE SERUM PROMOTES HUMAN MACROPHAGES TO ADHERE TO AND IMMOBILIZE *ASCARIS* LARVAE

To assess whether human blood monocyte-derived macrophages can attack *Ascaris* larvae, we performed an overnight co-culture of macrophages with newly hatched larvae. Given the similarities observed between *A suum* and *A lumbricoides*,[12] we set up these experiments using *A suum* larvae and immune serum collected from *A suum*-infected pigs. Human macrophages were able to recognize and adhere

to *A suum* larvae in the presence of immune serum (Figure 1A). In order to understand if cellular adherence to the larvae was dependent on complement alone, or whether other helminth-specific components of immune serum were required, we compared the percentage of attacked third-stage (L3) larvae by macrophages in the presence of naïve serum (NS) or immune serum (IS) (Figure 1B). Only immune serum was able to trigger significant larval attack by macrophages (total percentage of > 3 cells attacking per larva; L3 only: 6.61% ± 5.26, L3 and NS: 14.76% ± 7.54, L3 and IS 91.9% ± 6.73%). This indicates that complement alone may not mediate recognition. Using a previously established assay, we investigated whether the adherence of human macrophages to *A suum* correlated with a functional impact on larval motility. In brief, we assessed differences in larval shape between consecutive frames of time-lapse microscopy videos, and quantify motility using a customized macro. We observed that immune serum–activated macrophages significantly impaired larval motility (Figure 1C and D), indicating that these may contribute to protection against re-infection via larval trapping (39.7% average decrease in motility relative to larvae alone), mirroring what it has been shown in rodent models of helminth infection.[29,31,33,41] We observed a low level of intracellular Arginase-1 (Arg-1) on the human macrophages, which was unaffected by the presence of larvae or immune serum alone (data not shown). We next determined whether immune serum–mediated trapping of L3 *A suum* occurred through an Arg-1–dependent mechanism, by performing the macrophage-larval co-cultures in the presence of S-(2-boronoethyl)-L-cysteine (BEC), an arginase inhibitor. Whilst immune serum–activated macrophages could significantly impair larval motility, there were no significant changes observed in co-cultures with additional BEC (L3 + IS: 39.7% decrease, L3 + IS +BEC: 38.1% decrease; Figure 1D). In contrast to the rodent model *H polygyrus*,[41] inhibition of the high-affinity Fc gamma receptor I (FcγRI or CD64) using a blocking antibody failed to rescue larval motility following adherence by human macrophages (Figure 1E). Importantly, recent work by Iglesias et al demonstrated that porcine IgGs exhibit low binding affinity to human FcγRI, FcγIIa, FcγRIIb and FcγRIIIa,[42] which likely explains the inability of a CD64 blocking antibody to mediate any effect in this model. These data demonstrate that, unlike murine macrophages, human macrophages trap helminth larvae independently of Arg-1 activity.

3 | IMMUNE SERUM–MEDIATED LARVAL TRAPPING BY MACROPHAGES IS CONSERVED IN HUMAN *ASCARIS* INFECTION

We next sought to determine whether the similar mechanisms of cellular adherence and trapping could occur in *Ascaris*-infected humans. For this purpose, human macrophages were co-cultured with *A suum* L3 in the presence of serum previously determined to exhibit reactivity or not to *A lumbricoides* in an ELISA. Cellular adherence (Figure 2A) and larval motility (Figure 2B,C) were then assessed 24 hours later.

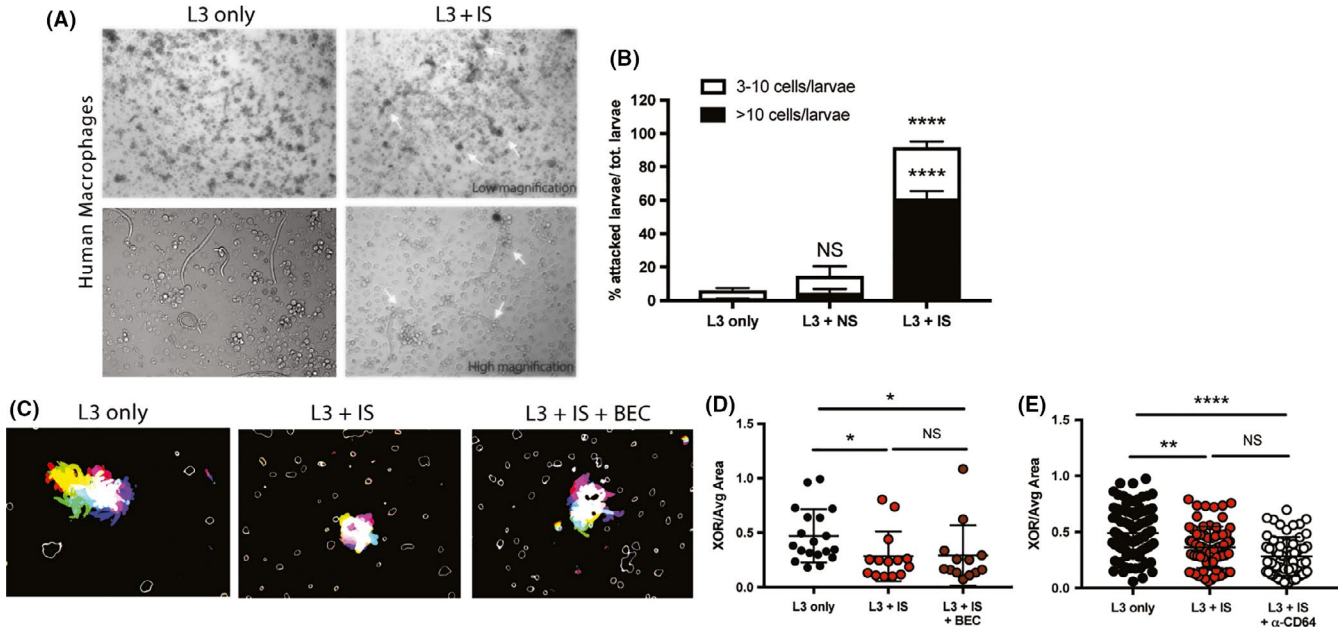

FIGURE 1 Immune serum–activated human macrophages bind to *Ascaris suum* larvae. A and B, *A suum* larvae (L3) were cultured with human macrophages for 24 hr in the presence of naïve serum (NS) or immune serum (IS) collected from *A suum*-infected pigs (1:50) and adherent macrophages per larvae was assessed by manual quantification using a light microscope. C, Representative temporal colour code time-lapse images of larvae incubated with human macrophages with or without IS ± 10 μm of the arginase inhibitor BEC (S-(2-boronoethyl)-l-cysteine) BEC. D and E, Larval motility (XOR/Average area) was quantified in human macrophages with or without IS ± 10 μm BEC ± 10 μg/ml anti-CD64 blocking antibody using our customized Fiji macro. The larval motility under each condition was normalized to the mean motility of larvae from co-cultures without serum. Data shown are pooled from at least three independent experiments and presented at mean ± standard deviation (Two-way ANOVA (B) or Kruskal-Wallis test (D,E) **P* < .05, ***P* < .01, ****P* < .001, *****P* < .001)

In keeping with our previous observations using *A suum* pig serum, *A lumbricoides* reactive human serum enhanced macrophage adherence to *A suum* L3 (Figure 2A: total percentage of > 3 cells attacking per larvae; L3 only: 5.88%, L3 and nonreactive serum: 20%, L3 and reactive serum 93.75%). Additionally, *A lumbricoides* reactive human serum also significantly enhanced macrophage immobilization of *A suum* L3 (52.5% average decrease in motility relative to larvae alone). Taken together, these data support our observations that parasite-specific antibodies facilitate macrophage adherence to larvae and subsequent larval immobilization, with this likely representing a conserved mechanism between pigs and humans.

4 | HUMAN MACROPHAGES EXHIBIT AN ALTERED GENE PROFILE FOLLOWING ACTIVATION BY ANTIBODIES AND *ASCARIS* LARVAE

In order to identify potential candidate genes in macrophages that could mediate the functional impairment of larval motility, we performed RNA sequencing. Macrophages, derived from differentiated blood monocytes of three donors (Donor1, Donor2, Donor3 in Figure 4A and B), were cultured for 24 hours in three different conditions: unstimulated 'control' macrophages ('ctrl'), macrophages stimulated with *A suum* L3 ('L') and macrophages stimulated with both L3 and pig immune serum ('LS'). We hypothesized that the co-stimulation given by the larvae and immune serum, which triggers larval adherence and trapping (Figures 1 and 2), would specifically induce the expression of genes involved in anti-parasite response. Initially, we employed principal component analysis (PCA) to generate a transcriptomic overview of differential macrophage treatments from normalized gene expression values (corrected for donor effect) (Figure 3A and Figure S1). The first principal component (PC1) explained 27.36% of the variance, showing that macrophages stimulated with both larvae and immune serum ('LS', triangle) clustered separately from unstimulated macrophages ('ctrl', square) or macrophages stimulated with larvae only ('L', circle). The variance was further confirmed by hierarchical clustering in the heat map reported in Figure 3B, which was generated using donor-corrected normalized expression of macrophages with larvae in presence or absence of immune serum, showing differential expression of 11 genes. We defined significantly differentially expressed genes as having a fold-change (FC) of >2 between two conditions using a *P*-value threshold set to control our false discovery rate (FDR) to 0.1. The 11 genes

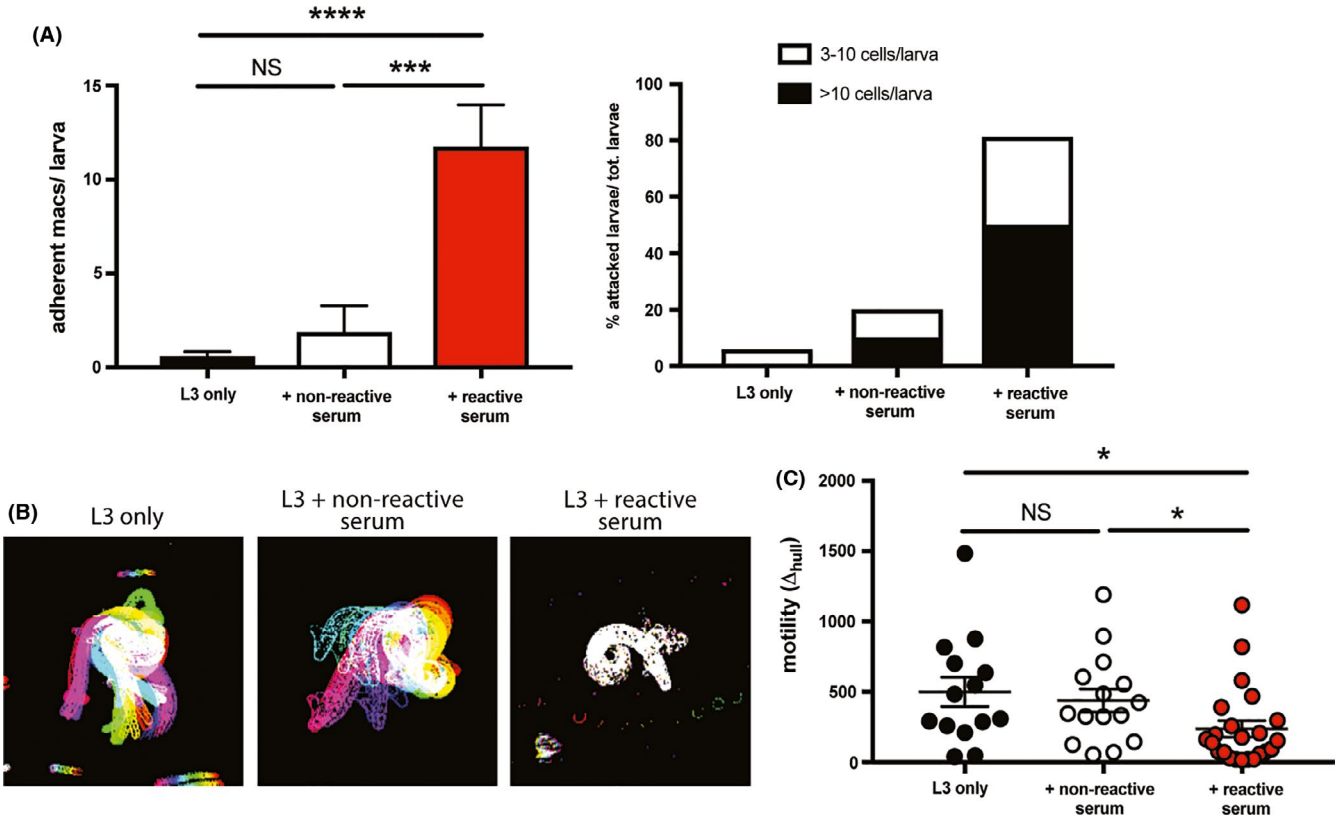

FIGURE 2 Immune serum from *Ascaris lumbricoides*-infected patients activates macrophages to impair motility of *Ascaris* larvae. A, Adherence of human macrophages to *A suum* L3 ± nonreactive serum or reactive serum from *A lumbricoides*-infected humans, as previously determined by ELISA, was assessed by manual quantification using a light microscope. B and C, Representative temporal colour code time-lapse images of larvae incubated with human macrophages ± healthy or patient serum (B) and quantification of larval motility using our customized Fiji macro (C). Data were normalized to the mean motility of larvae from co-cultures without serum. Data are pooled from at least two independent experiments and presented at mean ± standard deviation (Mann-Whitney *t* test (A) or one-way ANOVA, Kruskal-Wallis test (C), $^*P < .05$)

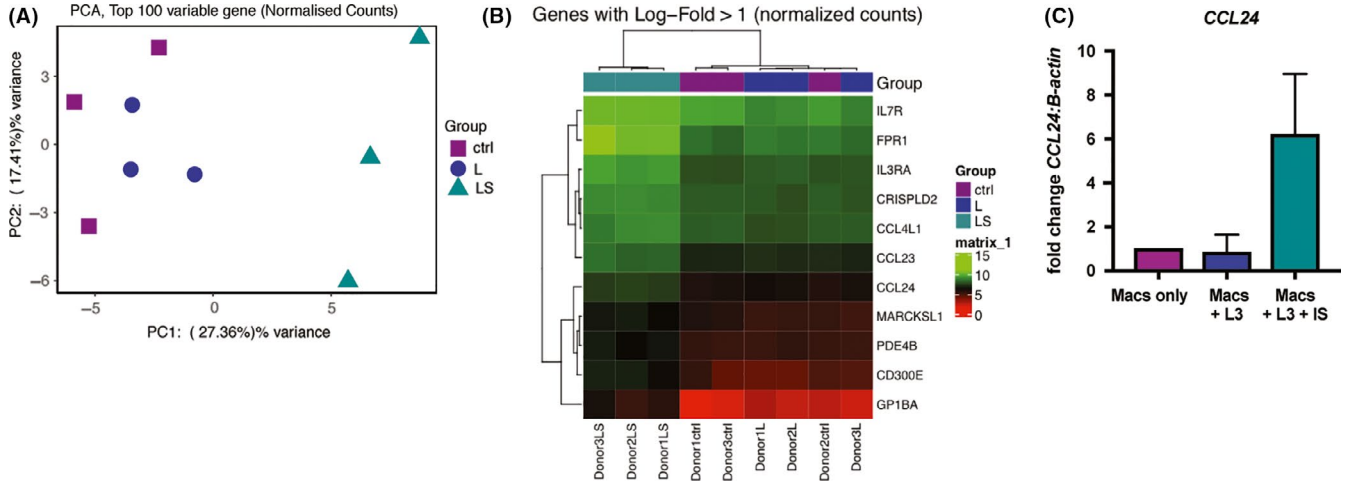

FIGURE 3 Human macrophages stimulated with *Ascaris suum* larvae and immune serum exhibit a distinct immune signature. Human macrophages from three separate donors were cultured in the presence *A suum* larvae ± immune serum collected from *A suum*-infected pigs (1:50). Cellular RNA was subsequently isolated and sequenced. Donorctrl: unstimulated macrophages; DonorL: macrophages + *A suum* larvae; DonorLS: macrophages + *A suum* larvae + immune serum. A, Principal component analysis (PCA) was performed between the three treatment conditions. Each condition is depicted with a different colour and shape. B, Heat map and hierarchical clustering of donor-corrected normalized expression data shows the 11 significant upregulated genes at false discovery rate (FDR) 0.1, cut-off: 2-fold-change. Red-green colour scale depicts gene expression (red: high; green: low). C, qRT-PCR validation of relative CCL24 expression in donor macrophages normalized to the reference gene β-actin

TABLE 1 Functional overview of genes displayed in the heat map of Figure 3B

Gene	FC↑	Function
CCL24	7.7	CCR3 ligand; eosinophil and basophil attractant, induced in murine M2 macrophages[73,77,78]
IL3RA	4.1	IL-3 receptor-alpha, it mediates cell survival and proliferation upon IL-3 binding[79]
IL7R	3.8	IL 7 receptor; involved in tissue-resident macrophage development and upregulated in response to LPS[80,81]
CRISPLD2	2.5	An LPS-binding protein, mediates inhibition of LPS-induced pro-inflammatory cytokines[43,44]
CCL23	2.5	CCR1 ligand; expressed in IL-4 stimulated human monocytes and involved in monocyte chemotaxis and adhesion[82,83]
GP1BA	2.5	Glycoprotein Ib platelet alpha subunit. also known as CD42b; potential role in M1 macrophage polarization[45,46]
FPR1	2.3	Formyl peptide receptor 1; involved in chemotaxis, killing of microorganisms through phagocytosis, generation of reactive oxygen species, induced in M1 macrophages[47,48,84]
PDE4B	2.3	Phosphodiesterase 4B; mediates LPS-induced TNFα production and IL-1Rα signalling and expression[49,50]
CD300E	2.3	Activating receptor on monocytes which induces pro-inflammatory responses upon ligation for example upregulation of TNFα, IL-8 and CD80/86[51,52]
MARCKSL1	2.1	Myristoylated alanine-rich C kinase substrate-like 1; involved in regulation of cell adhesion and migration, cytoskeletal reorganization, and membrane trafficking[85]
CCL4L1	2.0	Also known as MIP-1 beta; CCR5 ligand, redundant functions with CCL4, involved in chemotaxis and susceptibility to HIV[86]

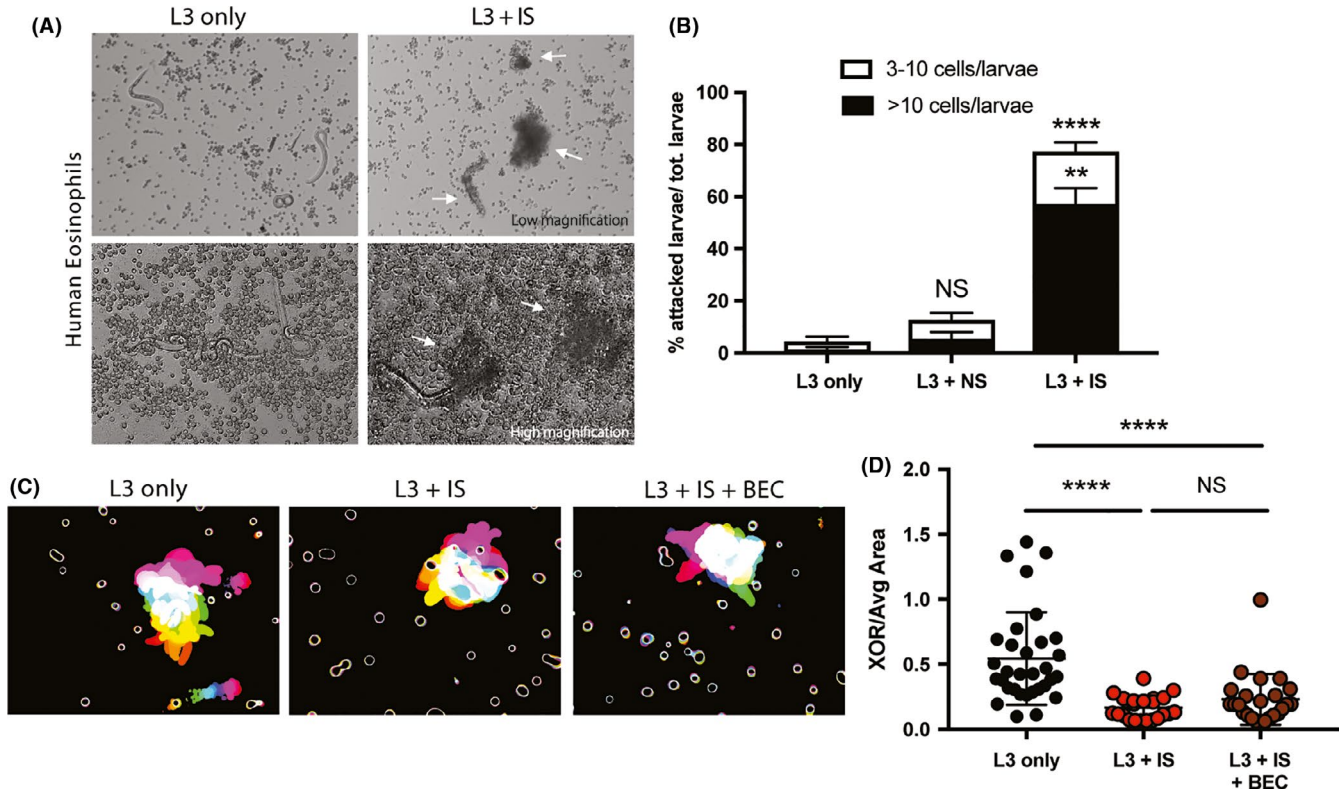

FIGURE 4 Immune serum–activated human eosinophils also bind and immobilize *Ascaris suum* larvae. A and B, *A suum* larvae (L3) were cultured with human eosinophils for 24 hours in the presence of naïve serum (NS) or immune serum (IS) collected from *A suum*-infected pigs (1:50) and adherent eosinophils per larva was assessed by manual quantification using a light microscope. C, Representative temporal colour code time-lapse images of L3 incubated with human eosinophils with or without IS ± 10 μm of the arginase inhibitor BEC (S-(2-boronoethyl)-l-cysteine) BEC. D, Larval motility (XOR/Average area) was quantified in eosinophil-larvae co-cultures with or without IS ± 10 μm BEC ± 10 μg/ml anti-CD64 blocking antibody using our customized Fiji macro. The larval motility under each condition was normalized to the mean motility of larvae from co-cultures without serum. Data are pooled from at least three independent experiments and presented at mean ± standard deviation (Two-way ANOVA (B) or Kruskal-Wallis test (D) $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .001$)

significantly altered in immune serum/larvae-treated macrophages are described further in Table 1. A closer look revealed a predominance of genes involved in the immune response. Some (eg *CRISPLD2*, *GP1BA*, *FPR1*, *PDE4B* and *CD300*[43-53]) are mainly upregulated in response to specific bacterial or inflammatory stimuli and appear to be more related to an M1 phenotype than the expected M2 phenotype.

Other genes encode for chemoattractants, such as CCL24, also known as eotaxin-2 or eosinophil chemoattractant protein 2. Notably, this chemokine was the most upregulated gene in macrophages stimulated with larvae and immune serum (7.7-fold increase). This suggests that one important function of macrophages may be the recruitment of eosinophils to the site of larval infection. QPCR analysis of *CCL24* expression in donor macrophages treated with larvae ± immune serum corroborates the findings from our global transcriptome analyses, showing elevated *CCL24* expression in treated with larvae and immune serum relative to controls (Figure 3C). Other interesting genes that were induced upon stimulation with immune serum and larvae and upregulated with a FC between 1.6 and 1.9 are linked to macrophage polarization (Table S1). Unexpectedly, however, they also indicate a more M1 polarized phenotype. In fact, both *SLC2A6* and *APOL3* have been shown to be more expressed in M1 compared to M2 macrophages,[54] whilst the contribution of the suppressor of cytokine signalling 3 (*SOCS3*) gene is thought to be an essential factor in supporting human M1 macrophage polarization.[55,56] However, there is also upregulation of other genes, such as Kruppel-like factor 4 (*KLF4*), which have been clearly associated with the M2 polarization.[57,58] As we were unable to identify specific candidates that may mediate the impairment larval motility, we cannot comment on particular effector mechanisms that orchestrate anti-parasite immunity in our in vitro model. However, we have been able to demonstrate that macrophages activated in the presence of larvae and specific antibodies express a distinct transcriptome and suggest a possible role for macrophages in mediating leucocyte chemotaxis during infection.

5 | IMMUNE SERUM–ACTIVATED HUMAN EOSINOPHILS EXHIBIT GREATER ABILITY TO IMMOBILIZE *A SUUM* LARVAE

Our previous data indicated that the human macrophages activated by immune serum plus *Ascaris* larvae not only functioned to immobilize larvae, but also produced CCL24 to recruit eosinophils. We therefore additionally tested the ability of immune serum to activate human eosinophils to recognize and immobilize *A suum* larvae.

Eosinophils were isolated from human blood and overnight co-cultures with L3 performed in the presence of immune serum (IS) or naïve serum (NS) from *Ascaris*-infected pigs. A large number of eosinophils were observed to quickly accumulate around and adhere to the larvae (Figure 4A,B; total percentage of > 3 cells attacking per larvae; L3 only: 6.85% ± 4.56, L3 and NS: 12.74% ± 11.1, L3 and IS 81% ± 11.9%). The efficiency of eosinophil-mediated attack in the presence of pig IS was similar to that observed for macrophages (eosinophils: 81% ± 11.9% and macrophages: 91.9% ± 6.73%; Figures 1B and 4B). Like macrophages, the response was dependent on immune serum, and activated eosinophils significantly impaired larval motility, albeit to a much greater extent than macrophages (eosinophils: 69.4% decrease in motility, and macrophages: 39.7% decrease in motility; Figures 1C and 4D, respectively). This observation is in keeping with previous studies reporting that porcine eosinophils can efficiently immobilize and kill *A suum* larvae in vitro.[38] No intracellular expression of Arg-1 was observed on the eosinophils (data not shown), and the addition of BEC to the cultures did not impact on the ability of immune serum–activated eosinophils to impair *A suum* L3 motility (L3 + IS: 69.4% decrease, L3 + IS +BEC: 57.8% decrease; Figure 4C,D). These data demonstrate that, like human macrophages, human eosinophils are activated by immune serum to adhere to, and trap, *Ascaris* L3, but also indicate that eosinophils may be more efficient than macrophages at this process.

Whilst the exact molecular components contained within immune serum that facilitate macrophage/eosinophil adherence to larvae remain unclear, likely candidates include specific antibodies, cytokines or a combination of both. As the IL-4 receptor signalling pathway has been previously reported to promote the adherence of murine macrophages to *N brasiliensis*,[31,59] we investigated whether IL-4 alone could promote the adherence of human macrophages and/or eosinophils to *Ascaris* larvae. Neither the adherence of human macrophages nor eosinophils in vitro to *Ascaris* larvae was affected by additional IL-4 stimulation in cultures containing larvae alone, naive serum or immune serum (Table S2), similar to previous observations made by Esser-von Bieren and colleagues in IL-4/serum co-cultures with *H polygyrus*.[29]

6 | METHODS AND MATERIALS

6.1 | Parasites

Ascaris suum adult worms were collected from intestines of infected pigs from a commercial slaughterhouse. *A suum* L3 were freshly hatched from embryonated eggs isolated from adult worm uteri following the protocol described by Masure et al (2013).[38] Briefly, eggs were incubated in sodium hypochlorite for 1 hour, washed three times with PBS and hatched by magnetic stirring with 2 mm diameter glass beads for 15 minutes. After overnight incubation at 37°C, the larvae were collected and washed three times with the following antibiotic mix: 10× penicillin/streptomycin (Thermo Fisher 10 000 U/mL), 3× gentamicin (Sigma 50 mg/mL), and tetracycline 30 μg/mL (Sigma). The larvae were then cultured in DMEM medium at 37°C for use in our in vitro experiments.

6.2 | Serum samples

Serum samples collected from *A suum*-infected pigs were collected as per approved ethics from the Ghent University EC2015/55. Patient serum was used from study participants in projects 032/2010 and 291/2008 recruited from the Western Cape, South Africa. Both projects were approved by the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee. Detection of IgG4 against *A lumbricoides* was tested by ELISA as per.[60] In brief, ELISA plates were coated with helminth 10 μg/ml for antigen-specific IgG4. Initial plasma sample dilutions were 1:50 (total IgG) and 1:20 (antigen-specific), followed by serial 1:5 dilutions of the initial dilutions. Secondary IgG (Southern Biotech) was used at 1:1,000, and secondary IgG subtypes (Southern Biotech) were used at 1:500. All secondary antibodies were alkaline phosphatase–linked. Healthy serum was defined as being IgG4 negative and patient serum as IgG4 positive as per.[60]

6.3 | in vitro generation of human blood monocyte-derived macrophages and eosinophil isolation

$CD14^+$ human monocytes and human eosinophils were isolated from buffy coats of healthy donors, in accordance with the Cantonal Ethics Committee of the Canton of Vauld (Vaud-Switzerland); written consent from the donors was obtained by the Lausanne blood transfusion centre, and they agreed that after absolute anonymity that certain components of their blood be used for medical research purposes. For CD14 + monocytes, magnetic separation was performed with CD14 + MicroBeads (Miltenyi Biotech) according to manufacturer's instructions. Briefly, PBMCs were isolated by density gradient using Ficoll-Paque (GE Healthcare; $\rho = 1.077$ g/ml). The leucocyte interphase was collected, washed and labelled with CD14 MicroBeads. After magnetic separation on LS columns (Miltenyi Biotech), cells were cultured for 7 days in complete RPMI medium (RPMI with 20% foetal bovine serum, 10 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin) with 20 ng/ml of human M-CSF (Peprotech). Eosinophil isolation was obtained with the human Eosinophil Isolation Kit (Miltenyi Biotec) according to manufacturer's instructions. Buffy coats were diluted in PBS and centrifuged on a Ficoll-Paque layer (GE Healthcare; $\rho = 1.077$ g/mL). The red cell pellet was lysed with the Red Blood Cell Lysis Solution (BioLegend). Cells were washed and magnetic labelling of noneosinophils with a biotinylated antibody cocktail and Anti-Biotin MicroBeads was performed. Subsequent magnetic separation with a LS column (Miltenyi Biotec) was performed and the eosinophil enriched fraction was collected and culture in complete RPMI medium. Cellular purity was checked by morphological analysis of stained cytospin preparations.

6.4 | Larval binding assay

Human macrophages (1×10^5) and eosinophils (2×10^5) were plated in a flat bottom 96-well plate; with technical triplicates performed

for each condition. Cells were then incubated with 50 *A suum* L3 larvae with or without serum collected from naive or *A suum*-infected pigs (1:50) for 24 hours at 37°C 5% CO_2. In some experiments, we added recombinant IL-4 (20 ng/ml, PeproTech) to the larvae/serum co-cultures. The percentage of larvae attacked by macrophages and eosinophils were then quantified using a stereo microscope with manual counting of live and motile larvae.

6.5 | in vitro motility assay

For each culture condition, motility of *A suum* larvae was assessed using a previously published protocol of time-lapse microscopy.[41] In our assays, human macrophages and eosinophils (1×10^6) were cultured for 24 hours with 500 *A suum* larvae and/or serum collected from naive or *A suum*-infected pigs (1:50) in a 12-well plate. In some experiments, we also added 10 μm BEC (S-(2-boronoethyl)-l-cysteine; Cayman Chemicals) or 10 μg/ml anti-CD64 antibody to the culture wells to decipher cellular mechanisms which may support larval attack. In other experiments, co-cultures were performed using human macrophages (1x106), 500 *A suum* L3 and reactive or non-reactive serum from A lumbricoides-infected patients (1:50). Time-lapse experiments were subsequently performed by recording movies of 60-s duration (120 frames of 0.5 s) with a CCD camera on a Olympus Cell R system (10×, 0.3 NA objective). Larval motility was quantified by using custom-made Fiji macro designed by Dr Romain Guiet of the Bioimaging and Optics Platform at EPFL (Switzerland) as detailed previously.[29]

6.6 | Real-time quantitative PCR

RNA of 1×10^6 cells was extracted with a Direct-zol RNA MiniPrep kit (Zymo Research), and reverse transcribed using RevertAid cDNA synthesis reagents (Thermo Scientific) for qPCR analysis. qPCR was performed using SYBR Green I Master Mix (Eurogentec) on an Applied Biosystems 7900HT System. Data analysis was performed with the Biomark HD Fluidigm, and expression was normalized according to expression of the housekeeping gene β-actin. Primers used in this study included the following: β-actin forward 5'-CTTTTCACGGTTGGCCTT-3', β-actin reverse 5'-CCCTGAAGTACCCCATTG-3'; and Arginase-1 forward 5'-GGCAAGGTGATGGAAGAAAC-3', Arginase-1 reverse 5'- AGTCCGAAACAAGCCAACGT-3'. Human CCL24 (ENSG00000106178) primer set is part of the PrimePCR Probe Assay (Bio-Rad Laboratories: qHsaCEP0040695]).

6.7 | RNA sequencing

Human macrophages of three donors (1, 2, 3) were derived from blood monocytes as described above. RNA was extracted from 1×10^6 cells per condition. RNA quality check, sequencing and data processing were performed at the Lausanne Technology Genomics Facility at the University of Lausanne. The RNA-seq pre-processing was performed in R (R version 3.1.2). Genes with low counts were filtered according to the rule of 1 count per million (cpm) in at least one sample. Library sizes were scaled using TMM normalization (EdgeR v 3.8.5; Robinson et al 2010) and log-transformed with limma voom function (R version 3.22.4; Law et al 2014) (see Figure S1). Differential expression was conducted using a linear model from the limma package. The linear model included a factor for the larvae presence, serum effect and each donor (paired analysis). The false discovery rate (FDR) was controlled for using the Benjamini-Hochberg (BH) method.

The following RNA-seq statistical analysis was performed in R (R version 3.6.1). Prior to principal component analysis (PCA), the TMM normalized expression data were sorted by greatest variance. The top 100 genes with greatest variance were selected for principal components calculation. PCA and visualization was performed on the normalized expression data using the R stats-package and ggplot2 (R version 3.2.1[61]). Significant genes with a 10% FDR and a log fold-change greater than 1 were selected from the comparison of macrophages stimulated with larvae and serum vs larvae. The normalized expression data of these genes were used to generate a heat map using ComplexHeatmap (R version 2.0.0[62]) with a Euclidean distance and Ward D clustering method.

6.8 | Statistics

Bars represent the mean, and the error bars represent the standard deviation. The Kruskal-Wallis test was used to compare three or more unmatched groups. *P*-values higher than 0.05 were considered nonsignificant. *P*-values lower than or equal to 0.05, 0.01, 0.001, 0.0001 are, respectively, represented by one, two, three or four asterisks (*). Statistical analyses were performed with GraphPad Prism 7 (GraphPad Software).

7 | DISCUSSION

The current study aimed to understand the role of human macrophages in mediating protective immunity against nematode parasites. We observed that human blood monocyte-derived macrophages could adhere to the surface *of A suum* L3 and impair their motility in response to activation by immune serum. Of special interest, human macrophages activated by immune serum and *A suum* L3 upregulated expression of the eosinophil chemokine *CCL24*. And eosinophils were observed to be significantly better than macrophages at trapping larvae. Tissue-resident macrophages are likely to be some of the first cells able to respond to invading larvae, and our data indicate that in immune individuals, these cells may serve a dual purpose in mediating protective immunity. In the first instance, they would recognize and adhere to the invading larvae, perhaps also impacting negatively on larval migration. Next, they

would secrete CCL24 prompting eosinophils to exit the circulation and migrate to the site of larval infection. At this point, macrophages and eosinophils would co-operate to immobilize, and eventually kill, the larvae interrupting larval migration and developing and halting the infectious cycle. Through the course of natural infection, L3 are found in the intestine of both *Ascaris*-infected humans and pigs.[14,22] Importantly, immunized *A suum*-infected pigs exhibited a strong eosinophilic response in the intestinal mucosa, limiting larval migration to the liver and lung.[22]

Although this model remains hypothetical, it is in keeping with histological studies from a wide range to host and parasite species indicating the presence of both macrophages and eosinophils in close proximity to tissue-migrating helminth larvae. It also coincides with findings in murine *H polygyrus* infection, whereby challenge-infection of mice leads to the rapid accumulation of Arginase-1 expressing macrophages around larvae in the intestinal serosa, with a later influx of eosinophils.[41] It is also supported by a recent study in *Brugia malayi*-infected mice, in which macrophages mediated immunity via recruitment of eosinophils to the peritoneal cavity in a CCR3-dependent fashion.[63] An increase in circulating CCL24 levels has also been reported in *Schistosoma mansoni* patients during acute infection and in patients given anti-helminthic treatment against *Oncocerca volvulus*.[64,65] Furthermore, CCL24 production was highly elevated in peripheral blood mononuclear cells (PBMCs) isolated from *Ascaris*-infected adults following exposure to *A lumbricoides* antigen extract.[66] Taken together, these data indicate that macrophages may often function to produce eosinophil chemoattractants after encountering parasitic larvae and that both cell types may act in concert to trap and/or kill the larvae.

Whilst *Ascaris* infection is classically associated with increases in both total and specific IgE, other antibody isotypes are also shown to be elevated.[13] Indeed, the concept of isotypic selection and balance may contribute to resistance, as serum IgG4 levels were shown to inversely correlate to worm burdens in Ascaris infection,[18] clinically symptomatic filariasis and onchocerciasis[67] and in patient cohorts treated with a recombinant vaccine against *Schistosoma japonicum*.[68] As such, the identification and selection of both specific antibody isotypes and candidate antigens represent an essential step in successful vaccine design. To this end, it was recently shown that mice vaccinated with crude *A suum* antigens rely on IgG1 and IgG3 subclasses for protection,[69] and vaccination against a 16-kDa protein expressed in both human and pig *Ascaris* was found to confer protection in experimental models of infection by inhibiting larval migration.[70,71] Interestingly, naïve serum was not sufficient to induce any larval recognition or trapping by human macrophages or eosinophils, suggesting that complement alone does not mediate this response. This was intriguing given a previous study that demonstrated the ability of porcine eosinophils to degranulate and kill *A suum* larvae in the presence of naïve serum, suggesting a mechanism which is partially complement-dependent.[38] These differences may highlight some fundamental distinctions in immune-effector mechanisms between host species during *Ascaris* infection. From our observations, it is yet unclear as to which components present in immune serum may activate human macrophages and eosinophils, with further work necessary to prove a specific role for complement, particular antibody isotypes, cytokines and/or other molecules which may contribute to this response. Whilst the addition of IL-4 to our serum co-cultures did not enhance binding of cells to larvae in vitro, previous studies have demonstrated that both IL-4 and IL-10 drive expression of CCL24 in both human monocyte-derived macrophages and murine bone marrow macrophages[72,73] and may suggest a role for these cytokines in driving a subsequent immune cascade through recruitment of eosinophils.

Although the mechanisms by which human macrophages exert protective potential remain unknown, we were able to rule out a requirement for Arginase-1. Whilst this contradicts what we observe in murine models of helminth infection, it is not surprising given the differences between human and murine macrophages. Although our study did not directly assess the possible mechanisms of eosinophil-mediated larval trapping, others have shown that these cells can target parasitic larvae via an array of mechanisms. Eosinophils can release extracellular traps, comprised of DNA fibres and eosinophil granule proteins to immobilize larvae of the ruminant parasite *Haemonchus contortus*.[74] Other studies have shown that eosinophil degranulation and the release of cytotoxic proteins, including major basic protein and eosinophil peroxidase, facilitate larval killing.[75,76] It is likely that human eosinophil granule proteins act as cytotoxic effector molecules against *A suum* larvae as this has been previously reported using porcine eosinophils.[38]

In summary, we have shown that human macrophages are able to adhere to, and trap, *A suum* larvae following their activation by immune serum. We have also shown that immune serum–activated macrophages secrete chemokines to attract eosinophils and that immune serum–activated eosinophils exhibit an even more potent capacity for larval trapping. As underlying mechanisms mediating macrophage trapping of larvae differed from that reported for rodent models of helminth infection, further studies will be required to understand the precise mechanisms by which these cells trap *Ascaris* larvae, and to determine the possible contribution of cell types other than macrophages and eosinophils, as well as a combination of other mediators including cytokines and complement. Determining the antibody isotypes and other factors involved in eliciting macrophage and/or eosinophil-mediated protection will also be informative as this could provide important information for the design of much needed protective vaccines against these insidious parasites.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

GC analysed and plotted data and wrote the manuscript. BV, TB and KS designed and performed experiments. AB analysed RNA-seq data. PG provided reagents and larvae. MH and WGCH provided human serum samples. JEvB and NLH designed and supervised the study and sought funding. NLH contributed to writing the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in [repository name tbd] at http ://doi.org/[doitbd], reference number [tbd].

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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