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ARTICLE

Hematopoietic cell-derived RELM α regulates hookworm immunity through effects on macrophages

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Abstract

Resistin-like molecule α (RELM α) is a highly secreted protein in type 2 (Th2) cytokine-induced inflammation including helminth infection and allergy. In infection with *Nippostrongylus brasiliensis* (*Nb*), RELM α dampens Th2 inflammatory responses. RELM α is expressed by immune cells, and by epithelial cells (EC); however, the functional impact of immune versus EC-derived RELM α is unknown. We generated bone marrow (BM) chimeras that were RELM α deficient (RELM $\alpha^{-/-}$) in BM or non BM cells and infected them with *Nb*. Non BM RELM $\alpha^{-/-}$ chimeras had comparable inflammatory responses and parasite burdens to RELM $\alpha^{+/+}$ mice. In contrast, both RELM $\alpha^{-/-}$ and BM RELM $\alpha^{-/-}$ mice exhibited increased *Nb*-induced lung and intestinal inflammation, correlated with elevated Th2 cytokines and *Nb* killing. CD11c⁺ lung macrophages were the dominant BM-derived source of RELM α and can mediate *Nb* killing. Therefore, we employed a macrophage-worm co-culture system to investigate whether RELM α regulates macrophage-mediated *Nb* killing. Compared to RELM $\alpha^{+/+}$ macrophages, RELM $\alpha^{-/-}$ macrophages exhibited increased binding to *Nb* and functionally impaired *Nb* development. Supplementation with recombinant RELM α partially reversed this phenotype. Gene expression analysis revealed that RELM α decreased cell adhesion and Fc receptor signaling pathways, which are associated with macrophage-mediated helminth killing. Collectively, these studies demonstrate that BM-derived RELM α is necessary and sufficient to dampen *Nb* immune responses, and identify that one mechanism of action of RELM α is through inhibiting macrophage recruitment and interaction with *Nb*. Our findings suggest that RELM α acts as an immune brake that provides mutually beneficial effects for the host and parasite by limiting tissue damage and delaying parasite expulsion.

KEYWORDS

bone marrow chimera, inflammation, lung, macrophage, parasitic-helminth

Abbreviations: AAMac, Alternatively activated macrophages; AMICA1, Adhesion molecule, interacting with CXADR antigen 1; ARG1, Arginase I; BAL, Bronchoalveolar lavage; BCL2, B-cell lymphoma 2; BM, Bone marrow; CCL19, Chemokine (C-C motif) ligand 19; CCL3, Chemokine (C-C motif) ligand 3; CCL6, Chemokine (C-C motif) ligand 6; CHIL3, Chitinase-like 3; DEG, Differentially expressed genes; EC, Epithelial cell; FBS, Fetal bovine serum; FCNB, Ficolin B; FGF18, Fibroblast growth factor 18; FPR-RS5, N-formylpeptide receptor; GSL, Griffonia simplicifolia lectin; IF, Immunofluorescence; JAM, Junctional adhesion molecule; L3, Infectious third-stage larvae; MIF, Macrophage migration inhibitory factor; MMP19, Matrix metalloproteinase 19; *Nb*, *Nippostrongylus brasiliensis*; PBS, Phosphate-buffered saline; PDGFRA, Platelet-derived growth factor receptor alpha; PFA, Paraformaldehyde; PS, Pathway score; RELM, Resistin-like molecule; RGS1, Regulator of G-protein signaling; SEM, Scanning electron microscopy; Th1, T helper type 1; Th2, T helper type 2; TM7SF3, Transmembrane 7 superfamily member; TREM2, Triggering receptor expressed on myeloid cells 2; WT, Wild-type

1 | INTRODUCTION

Infections with parasitic worms induce a T helper type 2 (Th2) immune response that is important for controlling parasite burdens during primary infection and for immunity to subsequent secondary infections.¹⁻⁴ Additionally, Th2 immune responses have evolved to rapidly repair tissue damage caused by parasitic worms, and to restore tissue integrity and homeostasis following parasite killing.⁵⁻⁷ However, excessive Th2 immune responses are detrimental to the host where they can contribute to allergic inflammatory responses and tissue fibrosis.⁸⁻¹⁰ Therefore, Th2 immune responses must be carefully balanced for optimal anti-parasitic immunity and tissue repair while

limiting excessive inflammation and fibrosis. Investigation of host factors that regulate such immune responses could have broad implications for the treatment of these pathologies. Here we investigated the contribution of Resistin-like molecule α (RELM α /R α) to this host regulatory pathway in helminth infection.

RELM α is a host-derived protein that is highly expressed in several disease conditions including helminth infection, colitis, diabetes, allergy, and asthma.^{11–16} In mouse models of asthma, RELM α expression is elevated in the lung following allergen challenge, where it was postulated to promote airway hyperresponsiveness.^{11,17,18} Other studies using genetic deletion of RELM α or RELM α overexpression have suggested instead a beneficial function for RELM α in limiting Th2 cytokine-induced inflammation in mouse models of asthma and mouse helminth infection.^{19–21} Although protective in dampening lung inflammatory responses, RELM α paradoxically impaired optimal parasite expulsion in infection with the hookworm *Nippostrongylus brasiliensis* (Nb).^{19,21}

The mechanism of RELM α -induced immunoregulation has been investigated in *in vitro* activated bone marrow (BM)-derived macrophages and dendritic cells.^{13,22–24} These studies showed that RELM α expressed by alternatively activated macrophages (AAMac) dampened CD4⁺ Th2 cell responses whereas RELM α derived from dendritic cells promoted CD4⁺ IL-10 production. However, whether *in vivo* derived RELM α from these immune cells functionally impacts helminth infection-induced inflammatory response or helminth expulsion is unclear. Indeed, RELM α is also expressed by non-immune cells such as airway epithelial cells (EC), although the function of non-immune cell-derived RELM α is less well understood. In contrast to immune cells that can traffic to various sites in the body, EC are stationary and provide a barrier against pathogens. Nevertheless, EC contribute to host protective immunity by secreting chemokines and other proteins, such as trefoil factors, that mediate lung tissue repair following hookworm infection.²⁵

In this study, we investigated the functional contribution of RELM α derived from immune and non-immune cells and explored the mechanism of RELM α inhibition of helminth expulsion. Employing RELM α -deficient BM chimeras, we show that immune cell-derived RELM α , and not EC-derived RELM α , downregulates the Th2 inflammatory response against hookworms and impairs clearance of worms by the host. Further, we identify CD11c⁺F4/80⁺ macrophages as the primary source of immune cell-derived RELM α in the lungs. We utilize CD11c⁺ macrophage-worm co-culture assays to demonstrate that RELM α impairs macrophage-worm interaction and killing. Last, to identify potential downstream mechanisms of RELM α signaling on macrophages, we utilized NanoString technology to measure RELM α -induced changes in expression of over 700 myeloid specific genes in purified lung macrophages. Functional enrichment pathway analysis revealed that RELM α treatment downregulated genes associated with macrophage-mediated helminth killing, such as cell adhesion and Fc receptor signaling, but upregulated genes associated with cell cycle and apoptosis and Th1 activation. Collectively, our data implicate immune cell-derived RELM α as an important regulatory factor in hookworm infection through two mechanisms: (i) inhibiting Th2

inflammatory responses and (ii) directly acting on macrophages to impair adhesion to the worm.

2 | MATERIALS AND METHODS

2.1 | Mice

C57BL/6 and CD45.1 mice purchased from the Jackson Laboratory were bred in-house. RELM α ^{-/-} (*Retnla*^{-/-}) mice were generated and genotyped as previously described.¹⁹ Mice were age matched (6 to 14 weeks old), gender matched, and housed five per cage under an ambient temperature with a 12 h light/12 h dark cycle. All protocols for animal use and euthanasia were approved by the University of California Riverside Institutional Animal Care and Use Committee (protocols A-20150028B and A-20150027E) and were in accordance with National Institutes of Health guidelines, the Animal Welfare Act, and Public Health Service Policy on Humane Care and Use of Laboratory Animals.

2.2 | BM transfer

C57BL/6 (CD45.2), CD45.1, and RELM α ^{-/-} CD45.2 mice were used in BM chimera generation. Age and sex-matched animals were used as recipients of BM isolated from wild-type (WT) or RELM α ^{-/-} mice. Recipients were sublethally irradiated twice with 600 rad and reconstituted with 3×10^6 total BM cells administered via retroorbital injection. Donor chimerism was evaluated 8 weeks later via flow cytometry using blood stained with allotype-specific antibodies that recognized CD45.1 or CD45.2 (eBioscience). Mice that showed <75% donor chimerism were excluded from further experimental analysis.

2.3 | Infection

Nb hookworms were obtained from the laboratory of Graham Le Gros (Malaghan Institute, New Zealand). Nb life cycle was maintained in Sprague-Dawley rats purchased from Harlan Laboratories (Indianapolis, Indiana). Mice were injected subcutaneously with 500 Nb infectious third-stage larvae (L3) and sacrificed at days 3, 7, or 9 post-infection. The number of parasite eggs in the feces of infected mice were counted using a McMaster counting chamber and saturated salt solution on days 6–9 following infection. To quantify the number of adult worms within the small intestine, the small intestines of infected mice were cut longitudinally and incubated in PBS at 37°C for 2 h to allow worms to migrate out of the tissue. The number of worms in the intestines were then manually quantified. To generate Nb immune mice, mice were allowed to clear Nb infection and re-infected with 500 L3 at 21 days post primary infection. Immune mice were sacrificed at day 4 post-secondary infection.

2.4 | Sample collection, processing, flow cytometry, and cell sorting

Bronchoalveolar lavage (BAL) fluid and cells were recovered through washing twice with 800 μ l of ice-cold 1X PBS. Cells were recovered by centrifugation and leukocytes were enumerated by manual

counting using a hemocytometer. For flow cytometry, BAL cells were blocked with 0.6 μg rat IgG and 0.6 μg anti-CD16/32 (2.4G2) and stained for 25 min with antibodies for SiglecF (E50-2440), Ly6G (1A8), MHCII (M5/114.15.2) (all; BD Biosciences, San Jose, CA); F4/80 (BM8), Ly6C (HK1.4), CD11b (M1/70), CD11c (N418), CD45.1 (A20), and CD45.2 (104) (all from eBioscience, Affymetrix; Santa Clara, CA). Cells were then washed and analyzed on an LSRII instrument (BD Bioscience), followed by data analysis using FlowJo v10 (Tree Star Inc.; Ashland, OR). FACS was conducted on Moflo Astrios instrument (Beckman; Brea, CA). Cell populations were identified as follows: alveolar macrophages (CD11c⁺F4/80⁺), dendritic cells (CD11c^{hi}MHCII^{hi}), eosinophils (CD11c⁻SiglecF⁺), monocytes (CD11b⁺Ly6C⁺), and neutrophils (CD11b⁺Ly6G⁺).

2.5 | RT-PCR

RNA from lung tissue was extracted with TRIzol (Sigma-Aldrich; St. Louis, MO), and RNA from cells was extracted by using the Aurum total RNA minikit (Bio-Rad; Hercules, CA). iScript reverse transcriptase was used for cDNA synthesis (Bio-Rad; Hercules, CA). RT-PCR was performed with the Bio-Rad CFX Connect system using Bio-Rad CFX Manager 3.1 software. *Retnla* and GAPDH primers were purchased from Qiagen (Hilden, Germany).

2.6 | Cytokine quantification

For sandwich ELISA, Greiner 96-well medium bind plates were coated with primary antibody to cytokines (RELM α , Peprotech (Rocky Hill, NJ); IL-13 and IL-4, eBioscience (San Diego, CA) overnight at room temperature. Plates were blocked with 5% newborn calf serum in 1X PBS for 1 h at 37°C. Sera or tissue homogenates were added at various dilutions and incubated at room temperature for 2 h. Cytokines were detected by applying biotinylated antibodies (RELM α , Peprotech; IL-13 and IL-4, eBioscience (San Diego, CA) for 1 h at 37°C followed by incubation with streptavidin-peroxidase (Jackson ImmunoResearch Laboratories (West Grove, PA)) for 1 h at room temperature. Substrate TMB (BD Biosciences, San Jose, CA) was added followed by addition of 2N H₂SO₄ as a substrate stop, and the optical density was captured at 450 nm. Samples were compared to a serial-fold dilution of recombinant cytokine.

2.7 | Histology

Lungs were inflated with 1 ml one part 4% paraformaldehyde (PFA)/30% sucrose and two parts OCT embedding medium (Fisher Scientific; Hampton NH) and stored overnight in 4% PFA/30% sucrose at 4°C. A total of 1 cm of the proximal jejunum was fixed in 4% PFA overnight at 4°C. Lungs and intestines were blocked in OCT and sectioned at 12 μm . For immunofluorescence staining, sections were incubated with rabbit anti-mRELM α (1:400, Peprotech; (Rocky Hill, NJ)), biotinylated *griffonia simplicifolia* lectin (1:400, Vector Laboratories; Burlingame, CA), CC10 (1:400, Santa Cruz Biotechnology; Dallas, TX), and F4/80 (1:400, eBioscience; San Diego, CA) overnight at 4°C. Sections were incubated with appropriate fluorochrome-conjugated secondary antibodies for 2 h at room temperature and counterstained

with DAPI. For pathology, lung sections were stained with H&E. Sections were visualized under a DM5500B microscope (Leica; Wetzlar, Germany).

2.8 | *Nippostrongylus*-lung cell co-culture

Lung tissue of naive or immune WT or RELM α ^{-/-} mice were cut finely, incubated in 30 $\mu\text{g}/\text{mL}$ DNase (Sigma; St. Louis, MO) and 1 mg/mL collagenase (Roche; Basel, Switzerland) for 25 min in a 37°C shaking incubator and passed through a 70 μm cell strainer to generate single cell suspensions. Cells were washed with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin (all from Gibco; Waltham, MA). Red blood cells were lysed by using ammonium chloride-potassium buffer (Gibco; Waltham, MA). CD11c positive and negative populations were obtained using CD11c positive selection microbeads (Miltenyi; Bergisch Gladbach, Germany). CD11c positive or negative cell fractions were incubated with 50–100 Nb L3 that were ex-sheathed with 0.25% NaOCl (Fisher Scientific; Hampton, NH) and washed in 400 $\mu\text{g}/\text{ml}$ neomycin (Fisher Scientific; Hampton, NH) and 400 U/ml penicillin-streptomycin (Gibco; Waltham, MA). Where indicated, CD11c⁺ RELM α ^{-/-} cell co-cultures were supplemented with 100 ng/ml recombinant RELM α protein. All treatments were supplemented with immune serum from secondary infected Nb-infected RELM α ^{-/-} mice and incubated at 37°C 5% CO₂. Co-cultures seeded with 0.2×10^6 cells were analyzed for cell adherence to worms and worm motility. Co-cultures seeded at 1×10^6 cells were analyzed for RELM α levels in culture supernatant and worm ATP assays. Supernatants were collected and frozen at -20°C for downstream analyses. Cell adherence to worms was measured with bright field images of 5 fields of view in each triplicate well of the co-culture assay. The number of cells attached per worm were manually quantified. Worm motility was measured by collecting 15 second videos of 5 fields of view of each well of each treatment. Motility was assessed with custom-made Fiji macros as previously described.²⁶ For worm measurements, larvae from co-culture assays or Nb-infected mouse lungs were placed on a thin layer of 2% agarose pad and covered by a coverslip. Images of worms were taken using a camera (Canon EOS T5i; Tokyo, Japan) attached to a bright field microscope (Leica DM2500; Wetzlar, Germany). Worm measurements were done by tracing worm length and width in bright field images. Worm length was determined by tracing the worm lengthwise long the central line. Worm width was measured by tracing the widest region of the midsection.

2.9 | ATP assay

At the end of the co-culture assay, cells adhered to worms were removed by lysing with water. Larvae from each treatment were individually picked in 1X PBS, mixed with CellTiter-Glo 2.0 luminescent reagent (Promega; Madison, WI) and homogenized using a bead beater at 4°C for 5 min. The homogenates were centrifuged at 1000 $\times g$ for 2 min. Supernatants were transferred to a black 96-well clear-bottom plate and luminescence was

recorded by using a Glomax Multi detection system (Promega; Madison, WI). An ATP standard curve was generated by using ATP disodium salt (Sigma-Aldrich (St. Louis, MO)).

2.10 | Scanning electron microscopy (SEM)

At day 7 of the co-culture assay, worms from each treatment were washed in 1X PBS and fixed in 2.5% glutaraldehyde (Fisher Scientific) for 1 h on ice. Samples were washed three times in 1X PBS and fixed in 1% osmium (Fisher Scientific; Hampton, NH) at room temperature in the dark for 1 h. Samples were washed three times in 1X PBS and dehydrated in an ethanol gradient; 25% ethanol for 10 min, 50% ethanol for 10 min, 75% ethanol for 10 min, and stored in 100% ethanol at 4°C until imaging. Samples were air dried overnight at room temperature and sputter coated in platinum and palladium. Samples were imaged using the XL30 scanning electron microscope (SEMTECH Solutions; North Billerica, MA).

2.11 | Gene expression analysis by NanoString

CD11c⁺ macrophages/dendritic cells were sorted from D9 *Nb* infected RELM α ^{-/-} mouse lungs using the Moflo Astrios sorter (Beckman; Brea, CA). A total of 8 × 10⁴ sorted RELM α ^{-/-} CD11c⁺ cells were plated overnight in media with 10% FBS, then serum-starved for 2 h in 1% FBS media. Cells were then stimulated for 4 h with control PBS or recombinant RELM α (200 ng) (*n* = 3 per group). Cell lysates equivalent to 5 × 10³ cells were prepared according to manufacturer's instructions and analyzed with Myeloid Innate Immunity v2 panel (NanoString; Seattle, WA). Gene expression analysis was conducted using the Advanced Analysis NanoString software. The NanoString Advanced Analysis algorithm generated biological pathway scores by using normalized expression and extracting pathway-level information from a group of genes using the first principal component (PC) of their expression data.²⁷ Data for each pathway were scaled across samples before taking the first PC by dividing each gene's log₂ expression values by the greater of either their standard deviation or 0.05. Pathway scores for PBS versus RELM α treatments were combined from two independent experiments and tested for statistical significance for a total of *n* = 6 per group. Pathway scores are represented as the difference in pathway score between the two treatments and heatmaps of gene expression. Gene expression ratios (mRNA counts of RELM α /PBS) of two independent experiments were separately calculated by the NanoString analysis software. Of the total 754 genes, differentially expressed genes (DEG) that reached *P* < 0.06 were investigated for putative functions in published studies or according to biology pathways (NanoString nCounter Advanced Analysis <https://www.nanostring.com/products/analysis-software/advanced-analysis> and KEGG Pathway <https://www.genome.jp/kegg/pathway.html>). Gene expression data was deposited on Geo (<https://www.ncbi.nlm.nih.gov/geo/>), GSE116552.

2.12 | Statistical analysis

All statistics were analyzed by Graphpad Prism software. Where appropriate, Student's *t* test (for normal distribution data), Mann-

Whitney nonparametric test (for asymmetric distribution data), and two-way ANOVA (for analysis of more than one experiment) were used (*, *P* ≤ 0.05; **, *P* ≤ 0.01; ***, *P* ≤ 0.0001).

3 | RESULTS

3.1 | *Nb* infection induces RELM α expression in EC and alveolar macrophages

RELM α exhibits a remarkably diverse expression pattern and is expressed by immune and non-immune cells in a variety of Th2 inflammatory diseases, including helminth infection and airway allergic inflammation. We and others previously showed that RELM α dampens in vivo Th2 immune responses to helminths, and that macrophage and dendritic cell-derived RELM α regulates CD4⁺ T cell cytokine expression.^{13,19,21} However, the contribution of RELM α derived from immune and non-immune cells in vivo following *Nb*-infection has not been evaluated. We examined cell-specific RELM α expression in WT C57BL/6 mice, subcutaneously injected with 500 *Nb* L3 (or control 1X PBS) and sacrificed at day 7 post-infection when RELM α protein levels in the infected tissues are highest.^{19,28} In accordance with previous studies, both the lung and small intestine, which are colonized by *Nb*, had significantly increased *Nb*-induced RELM α ; however, RELM α mRNA levels were over 400-fold higher in the lung (Fig. 1A). RELM α immunofluorescent (IF) staining of the lung and small intestine (Fig. 1B) revealed that RELM α was expressed in the EC lining the airway (green arrow) and in lung parenchymal cells (white arrow). In the small intestine, histological assessment of RELM α staining suggested that RELM α was expressed by goblet cells in the basal crypts (green arrow) and by circulating leukocytes in the submucosa (white arrow). Although RELM α expression in non-immune airway EC and intestinal goblet cells was possible to visualize according to cell morphology, identifying the specific immune cells that express RELM α by IF staining was not possible. Instead, we performed cell sorting on dissociated lung tissue of *Nb*-infected mice, where RELM α expression is highest, followed by RT-PCR analysis of RELM α mRNA levels, normalized to a housekeeping gene. Purity of sorted cells was analyzed by flow cytometry and H&E-stained cytopins, revealing >90% purity (Fig. 1C). CD11c⁺F4/80⁺ alveolar macrophages were the major immune cellular source of RELM α in the lung followed by CD11c⁺MHCII⁺ dendritic cells and CD11c⁻SiglecF⁺ eosinophils, whereas Ly6C⁺ monocytes and Ly6G⁺ neutrophils produce little to no RELM α (Fig. 1D). Together these studies reveal that *Nb* infection induces RELM α expression by lung and intestinal EC and by immune cells, specifically macrophages. To date, the contribution of each of these cellular sources of RELM α to the outcome of *Nb* infection has not been explored.

3.2 | Generation of RELM α ^{-/-} BM chimeras to determine the contribution of BM and non BM-derived RELM α in *Nb* infection

Given the lack of available cell-specific RELM α ^{-/-} mice, we took advantage of BM chimera technology to delineate the role of non-immune

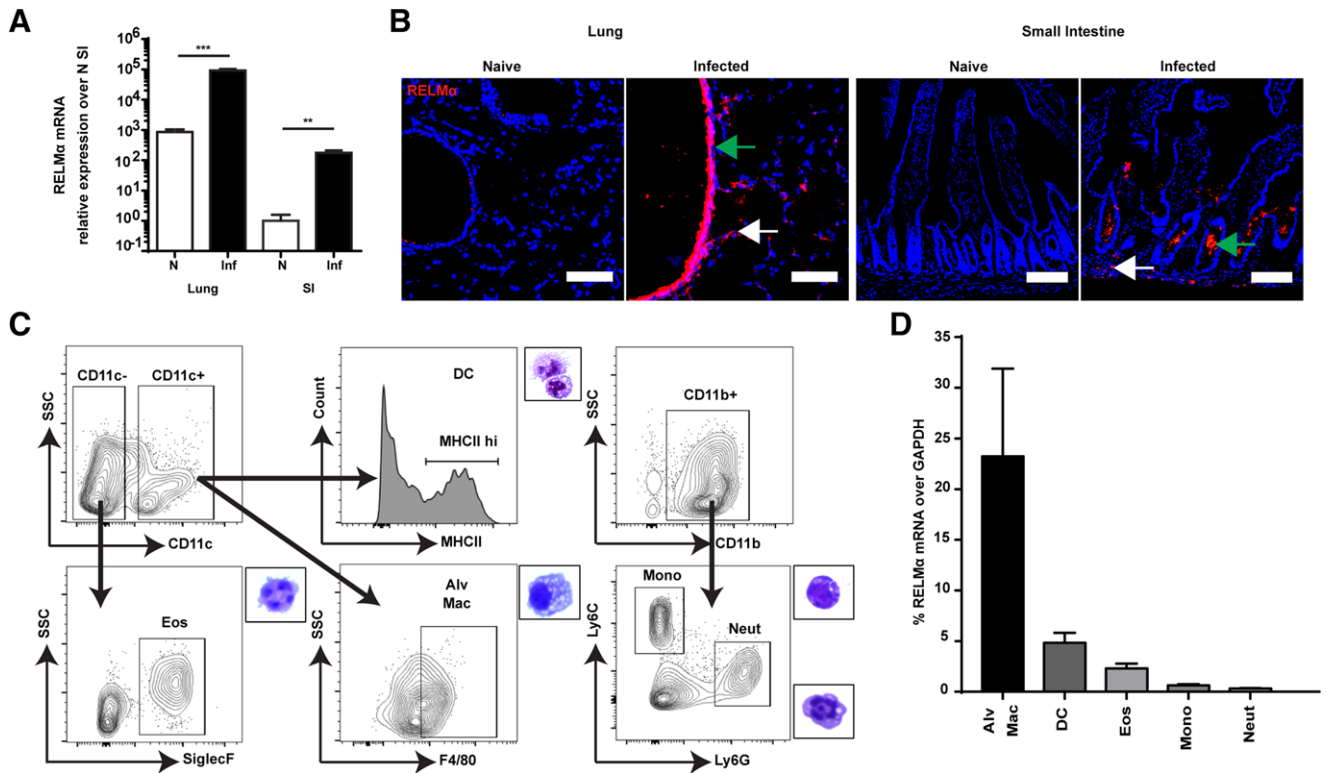


FIGURE 1 *Nb* infection induces RELM α expression in ECs and alveolar macrophages. C57BL/6 mice were left naïve or infected with *Nb* and evaluated for RELM α expression at day 7 post-infection. (A) RELM α RT-PCR in lung and small intestine (SI) was measured as relative fold induction over naïve SI ($n = 3$ mice per group). (B) IF staining of RELM α (red) and DAPI (blue) was performed in naïve and day 7 *Nb*-infected tissue. Lung; green arrow indicates airway cells and white arrow indicates parenchymal cells. SI; green arrow indicates basal crypts and white arrow indicates circulating leukocytes (scale bar, 100 μ m). (C) Gating strategy and confirmatory H&E cytospin for FACS sorted lung cells from *Nb*-infected mice. (D) RELM α mRNA expression was quantified in sorted alveolar macrophages (Alv Mac), dendritic cells (DC), eosinophils (Eos), monocytes (Mono) and neutrophils (Neut) as fold induction over neutrophils ($n = 4$ –6 mice per group). Data are represented as mean \pm SEM

and immune cell-derived RELM α in *Nb* immune responses. Recipient mice were irradiated to deplete BM, followed by retroorbital transfer of donor BM. Successful reconstitution was confirmed 8 weeks later by flow cytometric analysis of the peripheral blood for expression of the congenic markers CD45.1 and CD45.2 (Fig. 2A). Specific analysis of alveolar macrophages in the lung revealed greater than 96% reconstitution (Fig. 2B). BM chimeric mice were infected with *Nb*, and sacrificed at day 9, followed by examination of RELM α expression. Lung IF staining revealed RELM α co-stained with macrophage-binding lectin, *Griffonia simplicifolia* lectin (GSL) in WT and non BM RELM α ^{-/-} mice but not BM RELM α ^{-/-} or RELM α ^{-/-} mice, validating that BM-specific RELM α deletion abrogated macrophage expression of RELM α (Fig. 2C). Conversely, RELM α co-stained with airway EC marker CC10 in WT and BM RELM α ^{-/-} mice, but was abrogated when recipient mice were RELM α ^{-/-} (Fig. 2D). Similarly, we examined RELM α expression in the *Nb*-infected small intestine (Fig. 2E). All chimeras except RELM α ^{-/-} mice had RELM α ⁺ cells (red), but only WT and non BM RELM α ^{-/-} intestinal tissue sections had co-expression of RELM α with F4/80⁺ macrophages (green).

We next evaluated if deletion of RELM α specifically in the BM or the non-hematopoietic cell compartment affected local or systemic RELM α protein levels. BAL fluid RELM α was increased in *Nb*-infected WT mice compared to naïve WT mice (Fig. 2F). However, RELM α

deletion in the BM (BM RELM α ^{-/-}) did not affect BAL fluid RELM α levels, suggesting that BM cells contribute little to no RELM α in the airways. In contrast, when RELM α was deleted in airway EC (non BM RELM α ^{-/-}), BAL fluid RELM α was significantly reduced to levels observed in naïve mice. We observed the opposite phenotype for systemic RELM α expression (Fig. 2G), where BM-specific RELM α deletion significantly abrogated *Nb*-induced circulating RELM α , whereas non BM RELM α ^{-/-} mice still exhibited elevated serum RELM α levels. Together, these data delineate the contribution of immune and non-immune cell-derived RELM α in *Nb* infection, and show that RELM α in the airways is derived uniquely by non-immune cells, whereas immune cells are the main source of systemic RELM α in the serum. Nonetheless, the functional impact of local versus systemic RELM α in *Nb* immune responses is unknown.

3.3 | BM-derived RELM α regulates *Nb* infection-induced tissue inflammation, Th2 immune responses, and parasite expulsion

To elucidate the role of BM and non BM RELM α in *Nb*-induced lung and intestinal inflammation, histological examination of lung and intestinal tissue sections from infected BM chimeric mice was performed. RELM α ^{-/-} and BM RELM α ^{-/-} mice exhibited the most

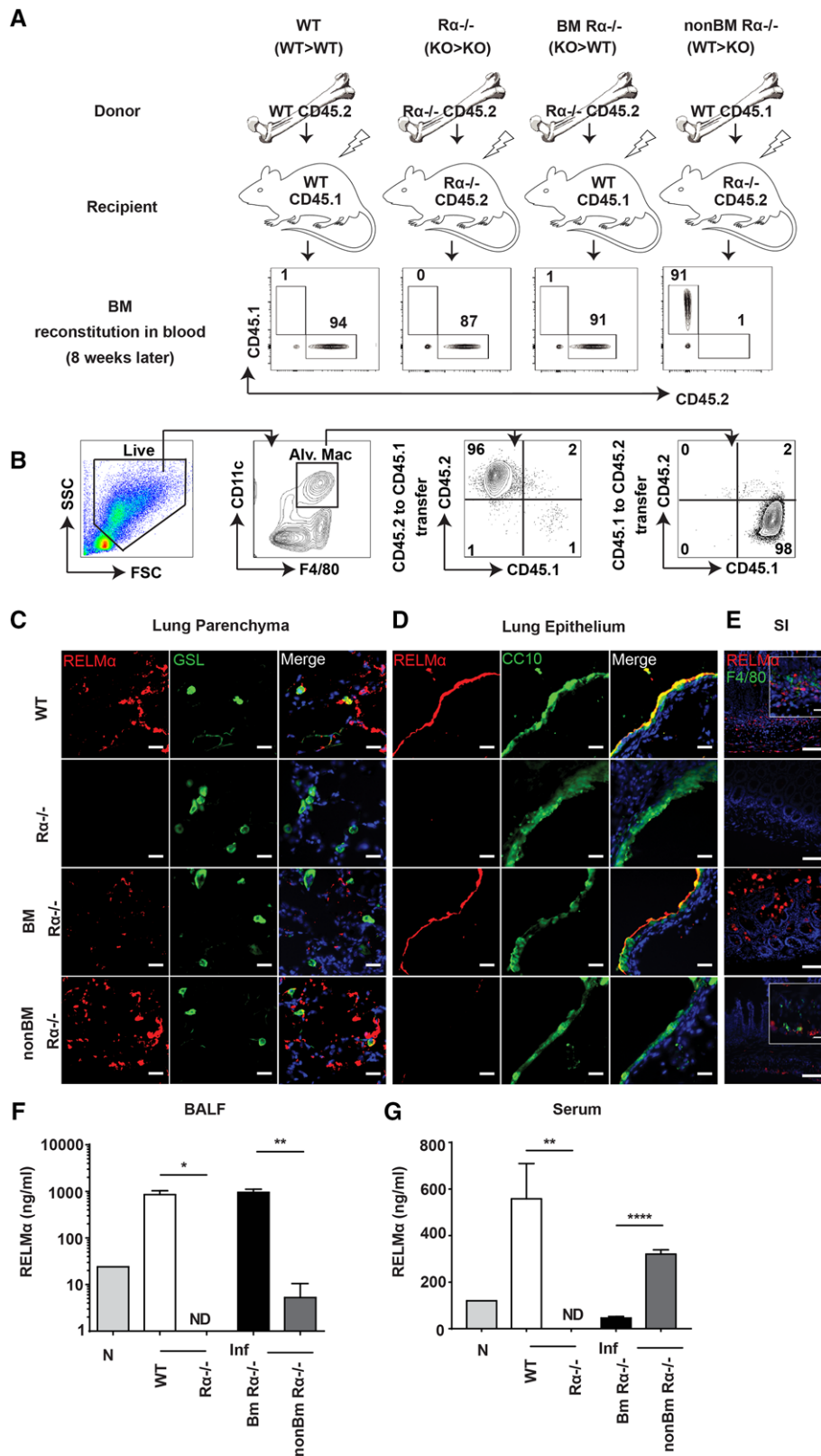


FIGURE 2 Generation of RELM $\alpha^{-/-}$ BM chimeras to determine the contribution of BM and non BM-derived RELM α in *Nb* infection. (A) BM chimera design and validation by flow cytometric analysis of peripheral blood. (B) Evaluation of alveolar macrophage reconstitution in bone marrow chimeric mice. (C-E) Day 9 *Nb*-infected BM chimeras were evaluated for RELM α expression by IF staining followed by microscopic examination. (C) Lung parenchyma and (D) lung epithelium stained for RELM α (red), GSL/CC10 (green), and DAPI (blue) (scale bar, 25 μ m). (E) Small intestine stained for RELM α (red), F4/80 (green), and DAPI (blue) (scale bar, 200 μ m, insert 50 μ m). (F-G) RELM α levels in the BAL fluid (F) and serum (G) of day 9 *Nb*-infected BM chimeras were measured by ELISA (ND not detected, $n = 4$ mice per group). Data are presented as mean \pm SEM, and representative of three separate experiments

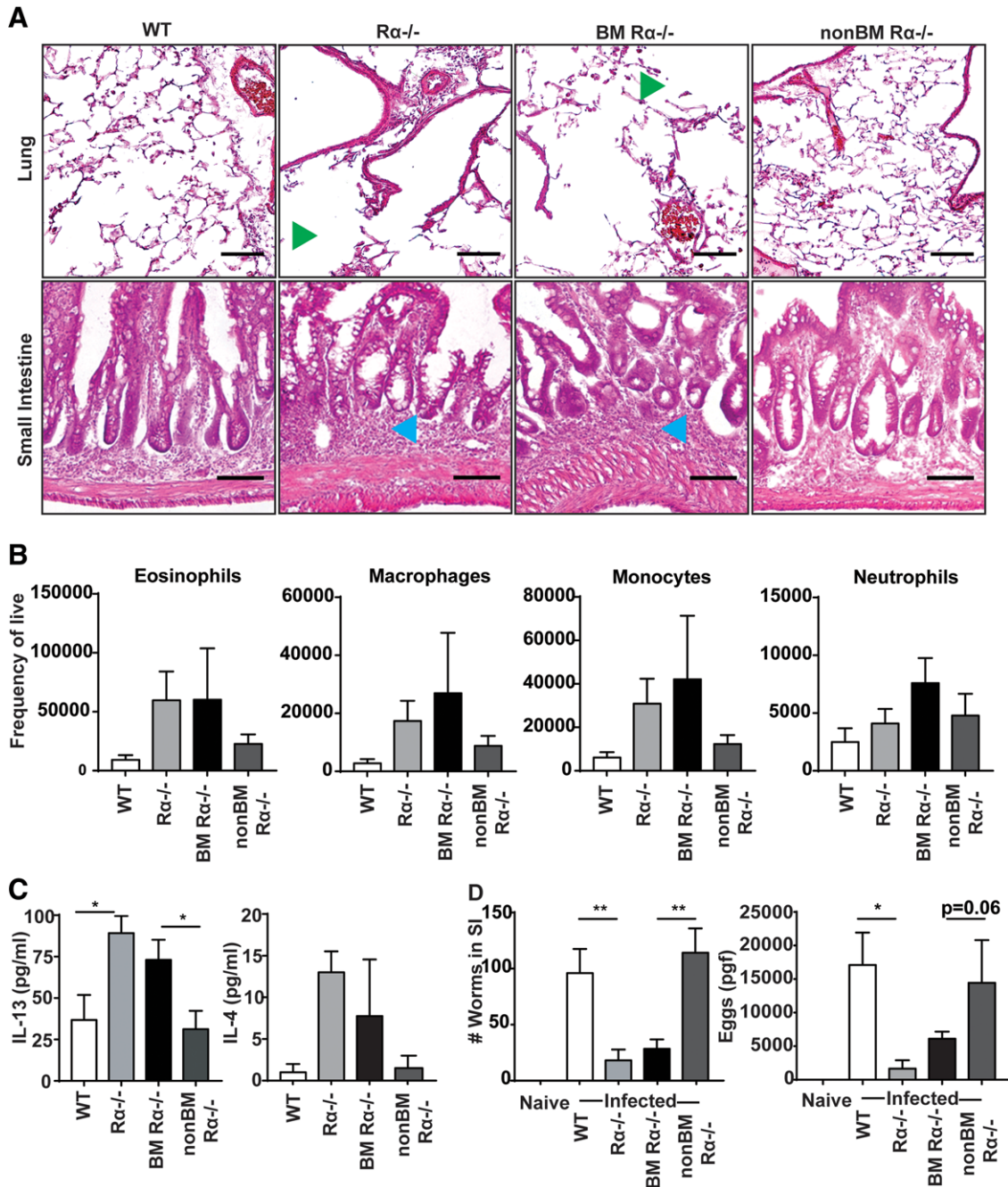


FIGURE 3 BM-derived RELM α regulates *Nb* infection-induced tissue inflammation, Th2 immune responses, and parasite expulsion. BM chimeras were infected with *Nb* and sacrificed at day 9 to evaluate *Nb* immune responses and burdens. (A) H&E stained sections of *Nb*-infected lung and SI tissue (green arrow, airway destruction; blue arrow, infiltrating leukocytes; scale bar, 200 μ m). (B) Flow cytometric analysis of BAL cell populations in infected mice ($n = 3-4$ mice per group). (C) IL-13 and IL-4 protein levels were measured by ELISA in serum and BAL fluid respectively of infected mice ($n = 3-6$ mice per group). (D) Intestinal worm and fecal egg burdens were measured at day 9 post-infection ($n = 8-21$ mice per group). For panel D egg burden, log transformation of the raw data was done prior to statistical analysis. Data are presented as mean \pm SEM

severe lung alveolar destruction (green arrow) and intestinal immune cell infiltration in the submucosa (blue arrow) (Fig. 3A). In contrast, non BM RELM $\alpha^{-/-}$ had comparable tissue inflammatory responses to WT mice, suggesting that BM-derived RELM α critically protects from *Nb*-induced tissue damage and inflammation, whereas non BM-derived RELM α is dispensable. Flow cytometric analysis of the BAL cells revealed that there were no significant differences in immune cell populations; however, RELM $\alpha^{-/-}$ and BM RELM $\alpha^{-/-}$ mice

exhibited a trend toward increased Th2 inflammation including elevated eosinophils and macrophages compared to WT and non BM RELM $\alpha^{-/-}$ mice (Fig. 3B). Similarly, serum IL-13 levels and BAL fluid IL-4 levels quantified by ELISA revealed that RELM $\alpha^{-/-}$ and BM RELM $\alpha^{-/-}$ mice had elevated levels of these Th2 cytokines (Fig. 3C).

We next determined if the increased infection-induced inflammatory responses functionally impacted *Nb* burdens (3D). RELM $\alpha^{-/-}$ and BM RELM $\alpha^{-/-}$ mice had significantly reduced intestinal worm and

fecal egg burdens, whereas there were no differences between WT and non BM $RELM\alpha^{-/-}$ mice. Therefore, although $RELM\alpha$ is highly expressed by non BM-derived airway EC and BM-derived immune cells, $RELM\alpha$ from immune cells is necessary and sufficient to down-regulate *Nb* immune responses, whereas non BM-derived $RELM\alpha$ has no obvious effect on *Nb* infection. Functionally, BM-derived $RELM\alpha$ is host-protective by limiting tissue damage and inflammation, but also leads to higher parasite burdens likely due to impaired Th2 cytokine-mediated mechanisms of *Nb* killing.

3.4 | $RELM\alpha^{-/-}$ CD11c⁺ lung macrophages have enhanced ability to bind and impair *Nb* fitness

Previous studies using a *Nb* vaccination model have shown that AAMac from the lung interact with and mediate *Nb* killing.²⁹ Together with our findings that $RELM\alpha$ deficiency specifically in immune cells enhanced *Nb* killing, we hypothesized that $RELM\alpha^{-/-}$ macrophages would exhibit enhanced ability to kill *Nb*. We, therefore, investigated whether $RELM\alpha$ affected lung macrophage interaction and killing of *Nb* L3 in an in vitro *Nb*-lung cell co-culture assay, modified from the *Nb* vaccination studies (Fig. 4A). WT and $RELM\alpha^{-/-}$ mice were infected with *Nb* for 21 days, followed by secondary *Nb* challenge to enhance AAMac responses. Four days following re-infection, lungs were recovered for isolation of lung macrophages. Lung alveolar macrophages express CD11c; therefore, we performed CD11c enrichment by magnetic bead purification. Although lung dendritic cells also express CD11c, the percentage of lung dendritic cells (CD11c⁺MHCII^{hi}, 20%) is lower than lung macrophages (CD11c⁺F4/80⁺, 60%). We first examined $RELM\alpha$ secretion by CD11c positive and negative fraction in response to co-culture with live *Nb* L3 (Fig. 4B). Co-culture with *Nb* L3 led to increased $RELM\alpha$ secretion especially in the CD11c⁺ fraction. These results are consistent with the RT-PCR results of sort-purified lung cells, and confirm that CD11c⁺ macrophages express more $RELM\alpha$ than other immune cell-types such as eosinophils, which have been previously reported to express high $RELM\alpha$ levels.

We next examined CD11c⁺ lung macrophage interaction with *Nb* L3 over the course of 7 days (Fig. 4C). There was equivalent cell adherence to the *Nb* at day 1 post co-culture; however, we observed that $RELM\alpha^{-/-}$ CD11c⁺ cells exhibited increased adherence to worms compared to WT cells starting at day 3 post co-culture, suggesting that $RELM\alpha$ inhibited the ability of CD11c⁺ cells to bind to *Nb*. To determine if cell adherence functionally affected *Nb*, we measured *Nb* motility in the co-culture using videos. Compared to *Nb* incubated with WT macrophages, *Nb* incubated with $RELM\alpha^{-/-}$ macrophages had significantly decreased motility (Fig. 4D). At the end of the in vitro co-culture, we recovered *Nb* L3 and measured worm ATP levels as a measure of worm viability (Fig. 4E). There was a significant decrease in *Nb* ATP levels from $RELM\alpha^{-/-}$ macrophage cultures compared to WT macrophage cultures. Together, these data suggest that $RELM\alpha$ inhibits macrophage adherence to *Nb*, and subsequent functional effects decrease *Nb* viability.

It is possible that the enhanced phenotype of $RELM\alpha^{-/-}$ lung macrophages is indirect as a consequence of the elevated in vivo Th2

cytokine response, which would promote AAMac activation. Alternatively, because WT macrophages secrete $RELM\alpha$ in vitro in response to co-culture with *Nb* L3, $RELM\alpha$ in the supernatant may directly regulate macrophage-*Nb* interaction. To delineate direct versus indirect effects of $RELM\alpha$, we supplemented $RELM\alpha^{-/-}$ macrophage cultures with recombinant $RELM\alpha$ and examined cell adherence to *Nb* and subsequent effects on *Nb* fitness. The addition of $RELM\alpha$ to $RELM\alpha^{-/-}$ macrophages partially decreased cell adherence, resulting in an intermediate phenotype between $RELM\alpha^{-/-}$ and WT macrophages (Fig. 4B). In contrast, $RELM\alpha$ treatment of $RELM\alpha^{-/-}$ cultures completely restored *Nb* motility and ATP levels to those observed in *Nb* cultured with WT macrophages (Figs. 4D–E). Together these results suggest that $RELM\alpha$ acts both directly on lung macrophages to suppress interaction with *Nb*, and indirectly, through other cell-types and cytokines to regulate macrophage activation.

We also examined *Nb* co-culture with CD11c⁺ cells from naïve (unvaccinated) WT or $RELM\alpha^{-/-}$ mice in comparison to co-culture with immune (vaccinated) CD11c⁺ mice (above). Naïve WT CD11c⁺ cells cultured with *Nb* produced significantly less $RELM\alpha$ than immune CD11c⁺ cells at days 3, 5, and 7 post co-culture (Fig. 4F). Examination of cell adherence to *Nb* revealed that both naïve WT and $RELM\alpha^{-/-}$ cells exhibited minimal binding (Fig. 4G). This was in contrast to immune cells, where $RELM\alpha^{-/-}$ CD11c⁺ cells adhered the most, consistent with previous findings (see Fig. 4C). Finally, immune $RELM\alpha^{-/-}$ CD11c⁺ cells were significantly better able to impair *Nb* motility than WT CD11c⁺ cells (Fig. 4H). However, no significant difference was found in unvaccinated WT or $RELM\alpha^{-/-}$ CD11c⁺ cells in their ability to reduce *Nb* motility. These results suggest that $RELM\alpha$ production and worm damage by CD11c⁺ cells require signals from the infection milieu in vivo.

To more closely examine the functional impact of $RELM\alpha^{-/-}$ cell interaction with *Nb* worms, we recovered *Nb* L3 from in vitro co-culture with WT or $RELM\alpha^{-/-}$ lung cells and measured worm size. *Nb* incubated with $RELM\alpha^{-/-}$ cells were shorter in length and significantly smaller in width compared to *Nb* incubated with WT cells (Fig. 5A). To visualize macrophage-*Nb* interaction, we performed SEM imaging of *Nb* L3 following co-culture with WT or $RELM\alpha^{-/-}$ macrophages (Fig. 5B). SEM images revealed close interaction and adherence of both WT and $RELM\alpha^{-/-}$ macrophages to *Nb* L3. However, WT macrophages were rounder, and the area of focal adhesion to the worm was small and distinct. In contrast, the focal contact point of $RELM\alpha^{-/-}$ macrophages appeared larger in area, resulting in flatter macrophages for a more expansive contact with the worm on a per cell basis. We investigated the physiologic relevance of the in vitro effects of $RELM\alpha^{-/-}$ cells on *Nb* growth in in vivo *Nb* infection. WT and $RELM\alpha^{-/-}$ mice were infected with *Nb* and sacrificed at day 3, followed by recovery of *Nb* larvae from the lungs. Although numbers of worms recovered from WT mice versus $RELM\alpha^{-/-}$ mice were equivalent (Fig. 5C), *Nb* recovered from $RELM\alpha^{-/-}$ lungs were shorter in length and significantly smaller in width compared to *Nb* recovered from WT lungs (Fig. 5D). Although there are important differences in timing between the in vitro (7 days) and in vivo (3 days), these data both indicate that *Nb*-induced macrophages differentiated in a $RELM\alpha$ -deficient environment exhibit an enhanced activation phenotype. We show that $RELM\alpha$

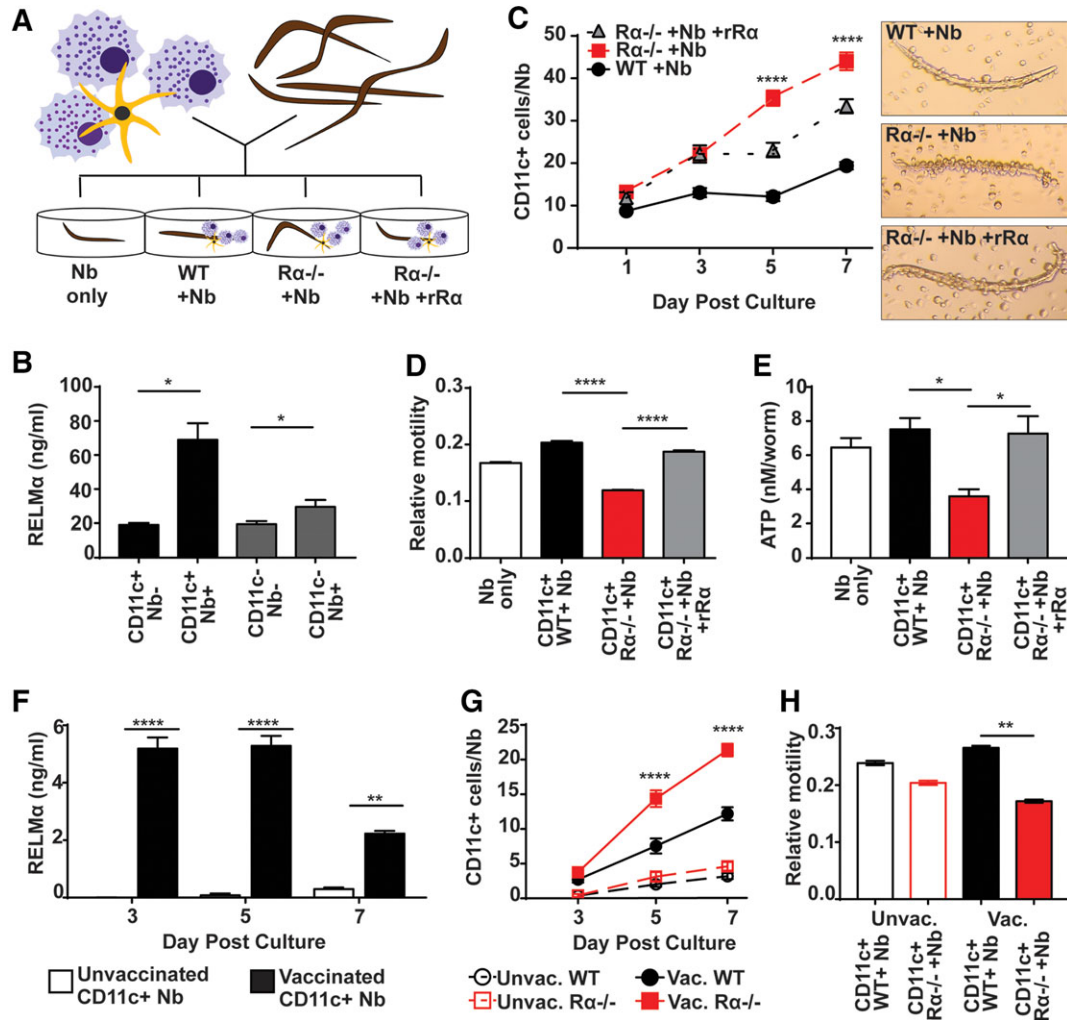


FIGURE 4 RELM α ^{-/-} CD11c⁺ lung macrophages have enhanced ability to bind and impair *Nb* fitness. (A) Design of co-culture assay of *Nb* L3 with lung cells from day 21 *Nb*-infected WT or RELM α ^{-/-} mice. (B) RELM α secretion by WT cells was measured in supernatants from day 3 post culture of CD11c positive and negative fractions from lung cells with or without *Nb*. (C) Microscopic quantification of adherent cells to *Nb* was performed for co-culture with CD11c⁺ cells from WT or RELM α ^{-/-} mice and representative bright field images shown. (D) Relative *Nb* motility (E) and ATP levels were measured at day 7 post co-culture. Where indicated, 100 ng/ml recombinant RELM α (rRa) was added to RELM α ^{-/-} CD11c⁺ cell-*Nb* co-culture. (F) RELM α secretion by unvaccinated versus vaccinated CD11c⁺ cells were measured at day 3, 5, and 7 post culture ($n = 3$ per group). (G) Microscopic quantification of adherent cells to *Nb* was performed for co-culture with CD11c⁺ cells from unvaccinated versus vaccinated mice and representative bright field images are shown. (H) Relative *Nb* motility was measured at day 7 post co-culture. Data are presented as mean \pm SEM ($n = 3$ /group), and representative of 2 separate experiments

directly inhibits macrophage-*Nb* interaction leading to impaired *Nb* killing. Because RELM α is predominantly expressed by macrophages, these data suggest that RELM α is secreted as an inhibitory cytokine that acts back on macrophages and other cell-types to dampen *Nb*-specific immune responses.

3.5 | Gene expression analysis reveals that RELM α signaling in lung macrophages downregulates pathways associated with cell adhesion and Fc receptor signaling

We previously showed that recombinant RELM α treatment of RELM α ^{-/-} macrophages could restore the WT macrophage phenotype, notably the reduced ability to bind to the worm and impair its

motility and fitness (see Fig. 4C). Therefore, we employed this controlled in vitro system to delineate potential downstream mechanisms by which RELM α regulates macrophage-*Nb* interaction. We utilized NanoString technology to screen over 750 myeloid-associated genes in RELM α ^{-/-} CD11c⁺ lung macrophages and identify those that were differentially expressed in response to RELM α . CD11c⁺ lung macrophages were sorted from the lungs of RELM α ^{-/-} mice at day 9 post *Nb* infection, with ~99% purity (Fig. 6A). Macrophages were rested overnight then stimulated with control PBS or recombinant RELM α for 4 h, followed by analysis of cell lysate for 750 myeloid associated gene-encoded mRNAs. NanoString advanced pathway analysis of RELM α versus PBS-treatment revealed a number of biologic pathways that were changed in response to RELM α treatment (Fig. 6B). Genes associated with Th1 cytokine and chemokine signaling,

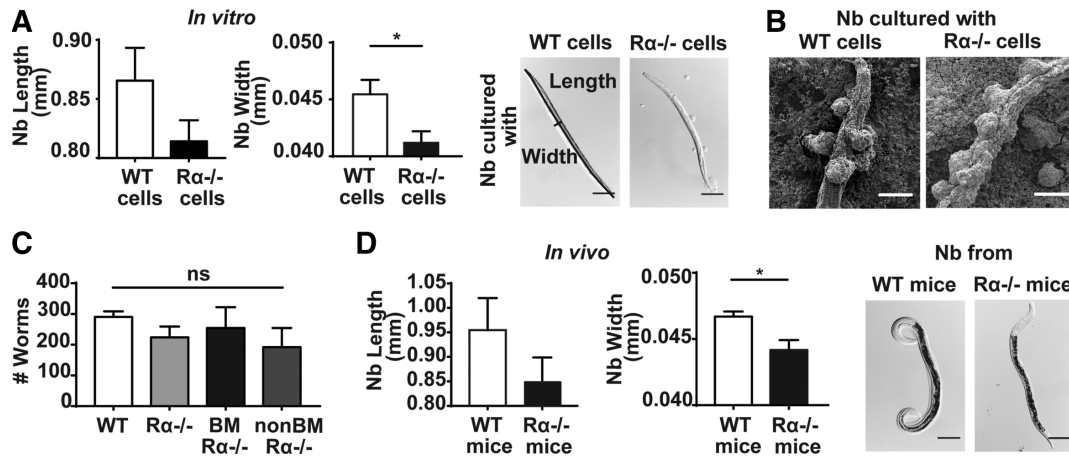


FIGURE 5 RELM $\alpha^{-/-}$ lung cells impair *Nb* growth. *Nb* from day 7 co-culture assay or day 3 infected lungs of WT and RELM $\alpha^{-/-}$ mice were compared. (A–B) *Nb* L3 worms from day 7 co-culture with total lung cells were measured and visualized for length and width (scale bar, 0.1 mm; $n = 9–11$ worms per group). (B) Scanning electron micrograph of *Nb* L3 and CD11c⁺ lung macrophages at end of co-culture (scale bar, 20 μm). (C) *Nb* larvae were collected from day 3 *Nb*-infected WT or RELM $\alpha^{-/-}$ mice, counted, and (D) measured and visualized for length and width (scale bar, 0.1 mm; $n = 5–6$ worms per group). Data are presented as mean \pm SEM

and cell cycle and apoptosis were upregulated. These results may be consistent with previous studies showing that RELM α promotes chemotaxis and proliferation.^{30–32} Given that RELM α downregulates Th2 cytokines,^{13,21} it is likely that Th1 cytokine signaling is conversely enhanced. In addition, genes associated with TLR signaling, antigen presentation, Fc receptor signaling, and cell migration and adhesion were significantly downregulated in the RELM α treatment compared to PBS (Figs. 6C and 6D). Downregulation of cell adhesion pathways is in line with our observation that RELM α treatment impairs cell adhesion to *Nb*. Further, previous studies have shown the importance of Fc receptor-mediated nematode killing;²⁶ therefore, downregulation of these pathways by RELM α may explain the reduced ability of macrophages to bind and impair *Nb* motility and fitness.

We further analyzed the differentially expressed genes between PBS versus RELM α treatments, using the cut-off value $P < 0.06$, and categorized them according to putative functions (Table 1). Of these, RELM α downregulated 14 genes and the remaining 18 were upregulated. Interestingly, some genes associated with alternatively activated or resolving macrophages activation were downregulated, such as *Arg1*, the macrophage inhibitory factor *Mif*, the anti-inflammatory receptors *Fpr-rs5* (member of the lipoxin receptor family N-formyl peptide receptor 1), and *Trem2*.^{33,34} Of the genes that were upregulated by RELM α , we found genes associated with wound healing (*Mmp19* and *Pdgfra*), genes associated with cell survival (*Tm7sf3*, *Bcl2*) and genes associated with macrophage signaling and effector functions (*Rgs1*). These results show that RELM α signaling impacts various biologic pathways and we have identified potential candidate genes that might be negatively regulated by RELM α to impair adhesion to the worm and killing.

4 | DISCUSSION

Although hookworms are intestinal parasites, their development relies on their initial migration through the host lung.³⁵ As such, the Th2

immune response that occurs in the lung is critical for parasite clearance, especially following secondary challenge, and needs to be considered when investigating protective immunity to hookworms.^{36,37} However, hookworm-induced lung inflammation must also be closely regulated to prevent aberrant worm-induced inflammation. Th2 cytokine-activated AAMac are critical contributors to this delicate balance between immunity and inflammation. In *Nb* infection, these cells can directly interact with and kill the worm but also are protective in resolving infection-induced lung hemorrhage and reducing neutrophil infiltration.^{5,29,38} AAMac also indirectly mediate *Nb* expulsion by promoting Th2 cytokine responses and inducing intestinal smooth muscle contractility.^{39,40} AAMac secrete factors and upregulate cell surface molecules that may contribute to these functions; however, studies delineating the contribution of these specific factors to AAMac function *in vivo* are lacking. In this study, we focused on the function of RELM α , a secreted protein that is highly expressed by AAMac in a Th2 cytokine-dependent manner.⁴¹

By utilizing BM chimeric mice, we tested the importance of BM-derived and EC-derived RELM α for the outcome of hookworm infection and hookworm-induced inflammation. BM-derived RELM α was found to downregulate immune cell infiltration in the lungs, IL-13, and IL-4 cytokines. Consequently, mice expressing RELM α only in BM-derived cells had higher worm burdens in the intestine compared to mice expressing RELM α in ECs. Therefore, we discovered that BM or immune cell-sourced RELM α is immunomodulatory whereas EC-sourced RELM α is not. An explanation for this observed phenotype could lie in the fundamental differences between immune cells and non-immune cells. Immune cells circulate in the blood between lymph nodes and inflamed tissue but in stark contrast, ECs are stationary cells. During an infection setting, immune cells such as AAMac have the capacity to communicate with other immune cells as well as interact with the parasite. These data are supportive of other studies showing immunoregulatory roles of AAMac during helminth infection. Whereas EC-derived RELM α is not immunomodulatory in *Nb* infection, high quantities of RELM α , presumably derived from EC, is observed in

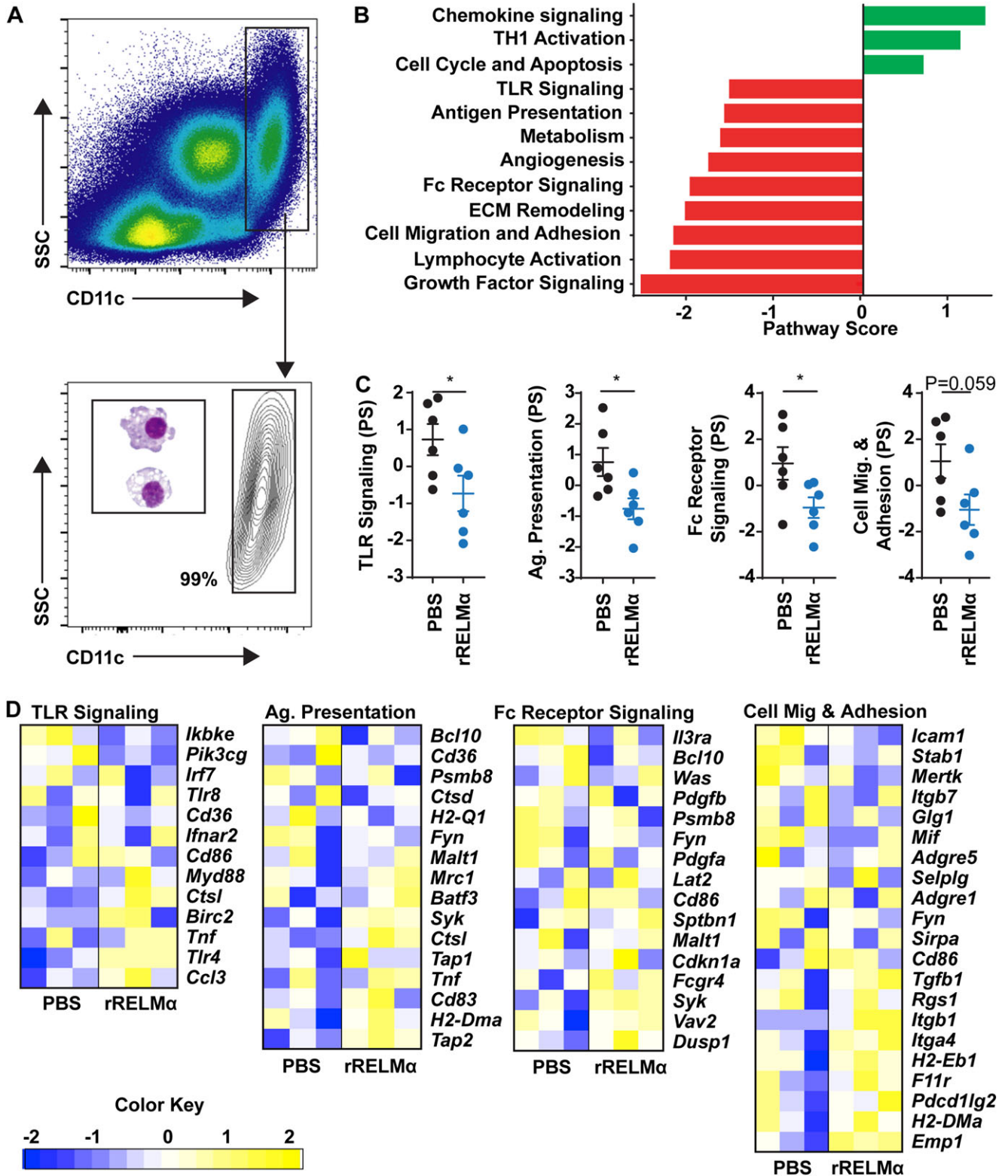


FIGURE 6 RELM α downregulates antigen presentation, Fc receptor signaling, TLR signaling, cell migration and adhesion. (A) CD11c⁺ macrophages were sorted from D9 Nb-infected RELM α ^{-/-} mouse lungs and evaluated for purity. Representative flow cytometry plot and cytopsin is presented. (B) NanoString analysis of biologic pathways that were changed by RELM α (n = 6 per group). (C) Biologic pathways downregulated by RELM α (n = 6 per group). (D) Heatmap showing individual gene expression of genes belonging to respective biologic pathway (n = 3 per group)

TABLE 1 Differentially expressed genes in PBS versus RELM α treatment. \dagger NanoString nCounter Advanced Analysis; $\#$ KEGG pathway

Putative function	RELM α suppressed	RELM α induced
Cell cycle and apoptosis		<i>Btg2\dagger</i>
Cell migration and adhesion	<i>Mif</i> \dagger , <i>Amica1</i> ⁶³ , <i>Ccl19</i> ⁶⁴ , <i>Ccr6</i> ⁶⁵ , <i>Fgf18</i> ⁶⁶ , <i>Fpr-rs5</i> ⁶⁷ , <i>Sema5a</i> ⁶⁸	<i>F11r</i> \dagger , <i>Itgb1</i> \dagger , <i>Rgs1</i> \dagger
Chemokine signaling	<i>Ccl19</i> $\#$, <i>Ccr6</i> $\#$	<i>Ccl3</i> \dagger , <i>Ccl6</i> \dagger , <i>Rgs1</i> \dagger
Lectin signaling	<i>Fcnb</i> ⁶⁹	<i>Irf1</i> $\#$
Cytokine/IFN signaling		<i>Tm7sf3</i> \dagger , <i>Il1r2</i> $\#$, <i>Irf1</i> \dagger , <i>Tnfrsf14</i> ⁷⁰
Differentiation and maintenance of myeloid cells		<i>Ikzf1</i> \dagger
ECM remodeling	<i>Adamts3</i> ⁷¹	<i>F11r</i> \dagger , <i>Furin</i> \dagger , <i>Itgb1</i> \dagger , <i>Kcnq1ot1</i> \dagger , <i>Mmp19</i> \dagger
Growth factor signaling		<i>Bcl2</i> \dagger , <i>Furin</i> \dagger , <i>Itgb1</i> \dagger , <i>Pdgfra</i> ⁷²
Lymphocyte activation	<i>Trem2</i> \dagger	<i>Golm4</i> \dagger , <i>Itgb1</i> \dagger
Metabolism	<i>Arg1</i> \dagger , <i>Sgpp1</i> \dagger , <i>Hnf1b</i> ⁷³	
Pathogen response/TLR signaling	<i>Rnase2b</i> ⁷⁴	<i>Bcl2</i> \dagger , <i>Chil3</i> \dagger , <i>Ccl3</i> \dagger

airways following allergen challenge. Whether EC-derived RELM α plays a more significant role in airway inflammation associated with asthma are avenues for future research.

Quantification of RELM α mRNA in sorted lung immune cells showed that alveolar macrophages were the principal source of RELM α in BM-derived cells. To further investigate the function of macrophage-derived RELM α , we performed co-culture assays of *Nb* with WT and RELM α ^{-/-} CD11c⁺ lung macrophages. We show that one mechanism by which RELM α impairs *Nb* killing is through impairing macrophage adherence and interaction with the worm. We employ recombinant RELM α to show that RELM α exerts its inhibitory effects directly on the worm or the macrophage. Previous studies investigating RELM protein binding of worms have shown that RELM β but not RELM α functionally affects helminth suggesting that the effect of RELM α may be on macrophages and not on *Nb*.^{42,43} In these studies, RELM β inhibited worm chemotaxis. Similarly, it is possible that RELM α inhibits macrophage chemotaxis to the worm. Alternatively, RELM α could regulate macrophage expression of surface integrins that mediate macrophage adherence.^{26,29}

To test which potential molecules could be regulated by RELM α , we performed Nanostring gene expression analysis of PBS or rRELM α -treated RELM α ^{-/-} lung macrophages sorted from *Nb*-infected mice. Among the RELM α -induced upregulated pathways were chemokine signaling and Th1 activation pathways. Our previous results showed lower Th2 cytokine expression; therefore, enhanced Th1 pathways in rRELM α -treated macrophages may be consistent with RELM α

regulating the balance between Th1/Th2 immune pathways. Within the chemokine pathway, RELM α upregulated *Ccl3* and *Ccl6*, while downregulating *Ccl19* and *Mif*. The differential effects of these chemokines are unclear, but may contribute to inhibiting macrophage migration to *Nb*. Consistent with our functional data showing that RELM α impaired macrophage adhesion to *Nb*, genes associated with cell adhesion were downregulated in RELM α -treated macrophages. Additionally, genes associated with Fc receptor signaling were downregulated, which may suggest RELM α -induced impairment of Fc receptor-mediated *Nb* killing.²⁶

Interestingly, expression of the AAMac-specific enzyme Arginase 1 was downregulated by RELM α ; however, the AAMac-secreted protein Ym1 (*Chil3*) was upregulated. Arginase 1 and its downstream products have previously been implicated in causing physical damage to *Heligmosomoides polygyrus* worms in vitro;²⁶ therefore, is a potential candidate gene for the reduced *Nb* viability when cultured with RELM α ^{-/-} macrophages. Other downregulated genes included *Fpr-rs5*, *Fcnb* and *Amica1*. *Fpr-rs5* is a member of formyl peptide receptor 1 family, which binds anti-inflammatory lipoxins and mediates biologic responses such as Ca²⁺ mobilization, cell attachment and migration.^{33,44} *Fcnb*, ficolin B, is a pattern recognition receptor that activates the complement pathway, which has been shown to mediate helminth killing.^{45,46} *Amica1* encodes a JAM transmembrane adhesion molecule that mediates myeloid cell migration and adhesion.⁴⁷ Lastly, *Fgf18* is a fibroblast growth factor that induces macrophage infiltration and M2 polarization.⁴⁸ Therefore, given their known functions, *Arg1*, *Fpr-rs5*, *Fcnb*, *Fgf18*, and *Amica1* are all RELM α -downregulated candidate genes, which could contribute to macrophage migration and adherence to worms.

For the RELM α -upregulated genes, we observed the matrix metalloproteinase *Mmp19* and the platelet-derived growth factor receptor *Pdgfra*. These genes encode factors associated with tissue repair, in line with RELM α 's proposed role in this process;⁴⁹ therefore, RELM α induction of these genes in vivo may contribute to enhanced lung tissue repair following *Nb*-induced acute injury.⁵⁰⁻⁵² RELM α also induced genes associated with cell survival, such as the apoptosis inhibitor *Bcl2* and *Tm7sf3*.⁵³ TM7SF3 is a seven-span transmembrane protein that protects from cellular stress and the unfolded protein response. Increased activation of this protein by RELM α may, therefore, promote cell survival. These findings are consistent with a previous study showing that RELM α inhibits apoptosis,¹¹ and suggest that RELM α preserves macrophage longevity. There are currently no known membrane receptors for RELM α , and future studies could investigate if RELM α binds TM7SF3 or a protein associated with this receptor. RELM α also induced expression of *Btg2*, p53-regulated gene associated with inhibiting proliferation.⁵⁴ This is contrary to previous studies showing that RELM α induces proliferation of endothelial and smooth muscle cell lines;^{55,56} however, the RELM α effects examined here were specifically in primary macrophages, which may explain these differences. Intriguingly, RELM α upregulated expression of *Rgs1*, a G-protein signaling regulator molecule, which has been demonstrated to reduce chemotaxis and dampen chemokine receptor signaling in macrophages and decrease integrin-dependent adhesion in B cells.⁵⁷ Together, our results suggest that RELM α inhibits macrophage

proliferation, promotes macrophage survival, and desensitizes macrophage effector functions. Of note, these gene expression changes were measured only 4 h post RELM α stimulation and represent macrophage-specific genes that are affected by cell-extrinsic RELM α , given that RELM $\alpha^{-/-}$ macrophages were used. Further in vivo studies are needed to delineate the direct and indirect effects of RELM α on macrophages compared to other cell-types. However, these gene expression analyses provide a useful foundation and candidate genes for investigation of the RELM α receptor and downstream signaling.

An interesting observation made in the co-culture assay was that *Nb* L3 cultured with WT macrophages were more motile and viable compared to *Nb* L3 alone. The improved fitness and activity of *Nb* L3 when cultured with WT cells could indicate that the worms require cues from the host for their activity and development. Studies of schistosomes have shown that the flukes require signals from host adaptive cells for their proper development.^{58–60} Similarly, it is possible that hookworms interact with and respond to host cells such as macrophages for their development. We found that *Nb* cultured with RELM $\alpha^{-/-}$ cells are less motile and viable compared to *Nb* with WT cells or *Nb* alone. This result could be due to significantly more immune cell damage to worms in the absence of RELM α . Our work is corroborated by previously published data that highlight the importance of macrophages and not dendritic cells in maintaining immunity to helminths.³⁹ However, in this study, macrophages were identified as CD11b⁺ cells and dendritic cells were identified as CD11c⁺ cells. In the *Nb*-infected lung, we found that macrophages co-express CD11c⁺ and CD11b⁺. One caveat of our methodology is that by purifying CD11c⁺ cells, we select for CD11c^{mid} lung macrophages and CD11c^{hi} dendritic cells. However, we find that alveolar macrophages are in higher frequency than dendritic cells in the lung and are the dominant cellular source of RELM α .

Given the results of the co-culture assay, we postulated that *Nb* isolated from RELM $\alpha^{-/-}$ lungs would have decreased fitness compared to WT mice. Length and width measurements of *Nb* confirmed this as worms from RELM $\alpha^{-/-}$ mouse lungs were smaller in size. These data highlight the significance of the lung immune response against the worm in weakening the parasite before it reaches the gut. We and others show that fewer adult *Nb* are recovered from the intestines of RELM $\alpha^{-/-}$ mice compared to WT mice.^{19,21} A recent publication was the first to show that *Nb* L3 feed on host red blood cells when in the lung and suggested that disrupting this feeding would lead to developmental arrest of the worm. Given the level of obstruction we see in co-culture assays where RELM $\alpha^{-/-}$ cells bind to and paralyze *Nb*, it is a possibility, that these L3s are then unable to feed and, therefore, suffer growth arrest.⁶¹ This is in line with our ex vivo observation that L4 recovered from RELM $\alpha^{-/-}$ mice seem developmentally impaired compared to those isolated from WT mice. Our data suggests that macrophages in the absence of RELM α damage the worm or prevent worm feeding leading to a functional impact on worm fitness and fecundity in the intestine.

In addition to high expression in the lung by hematopoietic and non-hematopoietic, RELM α derived from hematopoietic cells is also significantly increased in the blood of *Nb*-infected mice, and may modulate circulating immune cell function. Previous studies have shown

that helminth infection-induced AAMac can differentiate in the tissue, but also arise from circulating blood monocytes.⁶² In addition to the regulatory effects of RELM α on the macrophages in the lung, it is also possible that RELM α may exert regulatory effects on blood monocyte-derived AAMac that then infiltrate the infected lung and small intestine with potential functional consequences on worm killing or tissue inflammation. Indeed, we observed increased submucosal intestinal inflammation in the BM RELM $\alpha^{-/-}$ and RELM $\alpha^{-/-}$ mice, suggesting a regulatory role for RELM α in the intestine. The tissue-specific localization of macrophages, and the life cycle stage of the worm, may however determine their direct or indirect effects on the worm. *Nb* L3 and L4 are present in the blood and lung, respectively, and therefore are more likely to interact with macrophages. In contrast *Nb* adults reside in the intestinal lumen, and expulsion is dependent on several non-immune cells such as epithelial and smooth muscle cells that are nevertheless instructed by immune cells such as macrophages. Comparing RELM α 's functional effects on blood macrophages or intestinal macrophages versus lung macrophages could provide a more comprehensive understanding of the function of this pleiotropic molecule at different *Nb* life cycle stages.

In conclusion, our studies utilize BM chimeras and macrophage-worm co-culture assays to show that RELM α derived from macrophages downregulates *Nb*-induced inflammatory responses and subsequent *Nb* clearance partly by direct inhibition of macrophage-worm interactions. Macrophage-derived RELM α is therefore an immunomodulatory molecule that protects the host from an uncontrolled Th2 inflammatory immune response at the cost of killing parasitic worms. Delineating the protective versus pathogenic pathways triggered by macrophage-derived RELM α could provide therapeutic insight into tipping the balance toward host protective immunity against hookworms.

AUTHORSHIP

M.G.N., A.R.D., and H.M.B. conceptualized the study. H.M.B., J.L., G.C., D.L., and M.G.N. developed the methodology. H.M.B., G.C., D.L., J.L., J.J.P., J.C.J., K.C.R., and A.C.B. performed the investigation. H.M.B., G.C., J.L., D.L., K.C.R., and A.C.B. provided the formal analysis and visualization. H.M.B. and M.G.N. wrote the article. A.R.D. and M.G.N. supervised the study.

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DISCLOSURE

The authors declare no conflicts of interest.

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