Published in final edited form as:
Mucosal Immunol. 2014 September ; 7(5): 1094–1105. doi:10.1038/mi.2013.125.

A role for the pattern recognition receptor Nod2 in promoting recruitment of CD103+ Dendritic Cells to the colon in response to *Trichuris muris* infection

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Abstract

The ability of the colon to generate an immune response to pathogens, such as the model pathogen *Trichuris muris*, is a fundamental and critical defense mechanism. Resistance to *T.muris* infection is associated with the rapid recruitment of dendritic cells (DCs) to the colonic epithelium via epithelial chemokine production. However, the epithelial-pathogen interactions that drive chemokine production are not known. We addressed the role of the cytosolic pattern recognition receptor Nod2. In response to infection, there was a rapid influx of $CD103^+CD11c^+$ DCs into the colonic epithelium in wild type (WT) mice whereas this was absent in $Nod2^{-/-}$ animals. *In vitro* chemotaxis assays and *in vivo* experiments using bone marrow chimeras of WT mice reconstituted with $Nod2^{-/-}$ bone marrow and infected with *T. muris* demonstrated that the migratory function of $Nod2^{-/-}$ DCs was normal. Investigation of colonic epithelial cell (CEC) innate responses revealed a significant reduction in epithelial production of the chemokines CCL2 and CCL5 but not CCL20 by *Nod2*-deficient CEC. Collectively, these data demonstrate the importance of Nod2 in CEC responses to infection and the requirement for functional Nod2 in initiating host epithelial chemokine mediated responses and subsequent DC recruitment and T cell responses following infection.

Introduction

The gastro-intestinal dwelling parasite, *Trichuris muris* (*T. muris*), is a natural infection of mice and is also used as a model for the human parasite *Trichuris trichiura* (*T. trichiura*). *T. trichiura* infection affects over 1 billion people, with the highest prevalence being in developing countries[1]. Patients have a spectral immune response with some people immune to infection despite being in a disease endemic country, while others are susceptible

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Disclosures: None

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and harbour long term chronic infection[2]. This spectral immune response is also reflected in *T. muris* infection in mice. A strong Th2 response governs immunity to the parasite whereas a dominant Th1 response renders the host susceptible to infection.[2] Although the immune responses that govern resistance and susceptibility to *T. muris* are well characterised, how and why these immune responses are initiated is still unclear. Dendritic cells (DCs) are important cells for priming T cells and driving T cell subset polarisation[3]. Epithelial cells have been shown to play a critical role in promoting this ability of DCs to polarise T cell responses[4, 5]therefore, the epithelial/DC interaction may be an underlying factor as to why we observe differing immune responses to *T. muris* and indeed *T. trichiura*. *T. muris* is known to burrow into the epithelium of the large intestine and remains throughout its lifetime with its head end buried within an epithelial syncitial tunnel[2]. Given the close proximity of *T. muris* within the epithelial layer of the colon it is feasible that epithelial cells sense and respond to the parasite and initiate DC priming and immunity. Indeed, previous work has shown that immortalized intestinal epithelial cells (IECs) are able to respond to *T. muris* antigen[6] and work from our group demonstrated that resistance to infection is associated with the rapid recruitment of DCs, to the colonic epithelium via epithelial chemokine production [7]. However, the epithelial-parasite interaction that drives chemokine production and therefore DC recruitment is not known. Rapid recruitment of DCs to the colonic epithelium in *T. muris* infection was also associated with accelerated maturation of DCs[7] thus implying that DC recruitment to the epithelium is necessary for epithelial conditioning of DCs and induction of Th2 driven immunity.

Epithelial cells express several evolutionarily conserved and structurally related proteins called pattern recognition receptors (PRRs) that recognize specific microbe associated molecular patterns (MAMPs) such as lipopolysaccharide (LPS) or peptidoglycan (PGN) that are found on the surface of pathogens. PRRs also detect damage associated molecular patters (DAMPS) associated with tissue injury or cell death caused by inflammation and infection[8]. One such family of PRRs is the Nod-like receptors (NLR) that are primarily intracellular pattern recognition receptors located within the cytosol of cells[9]. The NLR Nod2 is a PRR of interest as mutations in the Nod2 gene have been associated with the inflammatory disorder Crohn's disease as well as increased susceptibility to infections[9]. The highest levels of Nod2 expression are found in epithelial cells and antigen presenting cells[10, 11], although Nod2 has also been identified in T cells[12] and neutrophils[13]. Within the colonic epithelium Nod2 expression is thought to be restricted to the dividing cells at the base of the crypts[14] which corresponds to the *T. muris* niche during the early phase of infection[2]. Nod2 specificity was originally thought to be restricted to the detection of muramyl dipeptide (MDP) on Gram positive and Gram negative bacteria, although now it is known to have a diverse role in host immunity. Nod2 has been attributed in virus recognition[15], T cell signalling[16], adaptive immune responses[17] and the regulation of host-microbiota crosstalk[18, 19]. However, a role for Nod2 in helminth immunity is yet to be defined. In this study we investigated the role of Nod2 in the initiation of the immune response to *T. muris*. We found that *Nod2*$^{-/-}$ mice had a reduced CD103^{+} DC recruitment to the epithelium. Furthermore, *Nod2*$^{-/-}$ mice had delayed parasite expulsion kinetics. Impaired DC recruitment was attributed to a reduction in colonic epithelial cell (CEC) responsiveness and chemokine secretion in response to infection. Our data implicates

a role for Nod2 in the initiation of immunity to infection via the regulation of CEC-derived chemokines and subsequent DC recruitment.

Materials and Methods

Mice

Male *Nod2*$^{-/-}$C57BL/6 mice have been described[20] and were bred in-house. Specific pathogen-free male C57BL/6 mice were purchased at 6–8 weeks of age from Harlan Olac (Bicester, UK). All mice were maintained by the Biological Services Unit (BSU), University of Manchester, UK and kept in individually ventilated cages. Animals were treated and experiments performed according to the Home Office Animals (Scientific Procedures) Act (1986).

Parasites

Maintenance of the *T. muris* lifecycle and production of excretory/secretory (E/S) antigen was carried out as described previously.[21] Mice were infected with approximately 175 embryonated eggs by oral gavage and sacrificed at various time points post infection (p.i.). Worm burdens were assessed as described previously[22].

ELISA

T. muris-specific IgG1 and IgG2a antibodies were measured in sera samples collected at autopsy by ELISA using a previously described method[22]. Chemokine production by colonic epithelial ells was measured by ELISA kit (R and D Systems, Abingdon, UK) for CCL2, CCL20, CCL5 and IL33 according to the manufacturer's instructions.

Histology

Caecal and colon snips were fixed in neutral buffered formalin (NBF) for 24 hours, processed and embedded in paraffin wax. 5 μm sections were then dewaxed, rehydrated and stained using a standard Haematoxylin & Eosin (H & E) or Periodic acid Schiffs (PAS) stain. Slides stained with H & E were measured for crypt hyperplasia, measured in 20 crypts per mouse using WCIF ImageJ software. Goblet cells were counted in 20 crypts per mouse from PAS-stained sections. All slides were measured and counted blind in a randomised order.

Immunofluorescence

Caecal and colonic snips were taken at autopsy and frozen in OCT embedding matrix (Thermo Fisher Scientific, Cheshire, UK). 6 μm sections were then fixed in 4% paraformaldehyde at 4°C for 5 minutes. Sections were blocked using the tryamide blocking kit (PerkinElmer, Cambridge, UK) for 30 minutes. Endogenous biotins were blocked using the avidin/biotin blocking kit as per the manufacturer's instructions (Vector Lab, Peterborough, UK). For four colour immunohistochemistry, slides were first stained with either purified anti-CD103 (Beckon Dickinson, Oxford, UK) and CD11b (Abcam, Cambridge, UK) or anti-F4/80 diluted in 0.1M Tris-HCL (pH7.5) (TNB). A secondary of mouse-anti-rat IgG2a-Cy5 or Anti-Rabbit AF-488 (Invitrogen, Paisley, UK) was applied to

slides. Slides were then incubated with the primary antibodies CD11c-biotin (Ebioscience, Hatfield, UK) and cytokeratin-FITC (Sigma Aldrich, Dorset UK). Samples were incubated with Streptavidin-Horseradish peroxidase for 30 minutes. After washing, samples were incubated with Tyramide Cy3 detection antibody for 5 minutes. Slides were washed and mounted with vector shield containing 4′,6-diamidino-2-phenylindole (DAPI, Vector Lab, Peterborough UK). Slides were imaged and $CD11c^+$, or $CD11c^+CD103^+$ or $CD11c^+F4/80^+$ counted per field of view in a blind randomized order. Three to four fields/view were counted per section.

Mesenteric Lymph Node cell culture and cytokine analysis

Single cell suspensions were prepared from mesenteric lymph nodes (MLNs) taken at autopsy and added at 5×10^6 cells per well in 1ml cultures to 48-well plates and stimulated with *T. muris* E/S at 50μg/ml. Cells were incubated at 37°C, 5% CO_2, 95% humidity for 48 hours, after which time supernatants were harvested and stored at −20°C. For cytokine analysis levels of IL-4, IL-10, IL-6, IL-9, IL-13, Interferon gamma, tumour necrosis factor α and IL-12p70 were determined using a custom cytometric bead array according to manufacturers instructions (CBA, Becton Dickenson, Oxford, UK) and analysed using BD FacsAria cytometer and FCAP Array software. For flow cytometry analysis single cell suspensions were prepared. Total cell numbers were counted and the cells were resuspended at 5×10^6 cells/ml. Cells were washed and Fc receptors were blocked using anti-CD16/32 (2 μg/ml E bioscience, Hatfield, UK). Cells were washed anti-CD4, anti-CD8, CD44, CD62L and anti-CCR9 (E bioscience) and acquired by flow cytometry on the BD LSRII. Data was analysed using FlowJo flow cytometry software (Tree Star inc. Oregon, US).

Generation of Bone marrow chimeras

Recipient mice were irradiated with two 5 Gy doses 4 hours apart and injected intravenously with bone marrow harvested from donor mice at 10 million cells /250μl sterile PBS. Bone marrow was allowed to reconstitute for 6 weeks before mice were infected with *T. muris*. Verification of successful reconstitution of bone marrow chimeras was determined by analysis of MAMP and MDP responsiveness of isolated bone marrow cells. Bone marrow was harvested and cultured as previously described[23]. On day six of the culture the cells were harvested from the plates and semi-adherent cells removed. The cells were spun at 400g and re-suspended at a concentration of 1×10^6 cells/ml in DC medium (RPMI 1640 supplemented with 10% LPS-free FBS (Gibco, Paisley, UK) 1% Penicillin/streptomycin and 50mM Beta-mercaptoethanol (Sigma Aldrich, Dorset UK)). The cells were stimulated overnight with LPS (100ng/ml) or MDP (5μg/ml). After 24 hours the cells were harvested and prepared for flow cytometry. FcR were blocked by incubating cells in anti-CD16/32 (2μg/ml) for 15 minutes. Cells were washed and stained with anti-CD45 PeCy7 (1 μg/ml, Becton Dickinson), anti-CD11c Alexa700 (1μg/ml), anti-MHC-II FITC (2.5 μg/ml) and anti-CD86 PE (1 μg/ml) (all E Bioscience) and acquired using a LSRII flow cytometer (Becton Dickinson Biosciences, Oxfordshire). Data was analysed using Flow Jo flow cytometry analysis software (Tree Star, Inc, Oregon, US).

Large intestine cell isolation and flow cytometry

Caecum and colon were harvested at autopsy and digested in RPMI containing 5% L-glutamine, 5% penicillin streptomycin, 10% fetal bovine serum (Sigma Aldrich, Dorset, UK), collagenase (1mg/ml), and dispase (0.5mgs/ml, both Gibco, Paisley, UK) for 2 hours at 37 °C. Cells were then forced through a 70μm cell strainer, centrifuged at 405g for 5 minutes and resuspended in 10mls 80% Percoll (GE Healthcare, Buckinghamshire, UK) solution which was then overlaid on a 40% Percoll solution. Cells were centrifuged for 25 minutes at 1000g and the cells at the gradient interface harvested. Fc receptors were blocked using anti-CD16/32 (2 μg/ml E bioscience, Hatfield, UK). Cells were washed and stained with anti-CD45 PeCy7 (1 μg/ml, Becton Dickinson, Oxford, UK), anti-CD103 PE (1 μg/ml), anti-CD11c Alexa700 (2.5 μg/ml), anti-MHC-II FITC (2.5 μg/ml) and anti-F4/80 APC (1 μg/ml) (all E bioscience, Hatfield, UK) and acquired by flow cytometry on the BD LSRII. Data was analysed using FlowJo flow cytometry software (Tree Star inc. Oregon, US).

Colonic and caecal cell isolation, cDNA conversion and qPCR

Caecum and colon were harvested at autopsy, digested in RPMI containing 5% L-glutamine, 5% penicillin streptomycin, 10% fetal bovine serum and dispase (1mg/ml Gibco, Paisley, UK)for 90 minutes at 37 °C. Cells were then forced through a 70μm cell strainer (Becton Dickinson, Oxford, UK) and spun at 400g for 5 minutes and resuspended in 1ml TRIsure (Bioline, London, UK). Epithelial purity was confirmed by flow cytometry using antibodies against CD326 (Ep-CAM, Cambridge Bioscience, Cambridge UK) and CD45-PE (Becton Dickinson). All epithelial preparations were >98% pure. Total RNA was isolated from cells by homogenizing in TRIsure, phases separated using chloroform (Sigma-Aldrich, Dorset, UK) and RNA precipitated in isopropanol (Sigma-Aldrich, Dorset, UK). RNA concentration was analysed on a nanodrop-1000 spectrophotometer (Labtech International, East Sussex, UK) and resuspended at a concentration of 1μg/μl using Bioscript – M-MLV kit (Bioscript, London, UK) for cDNA conversion. Quantitative PCR was performed using the SYBR green I core kit (Eurogentec, Southampton, UK) and an Opticon quantitative PCR thermal cycler (Bio-Rad, Hemel Hempstead, UK). Each sample was serially diluted, and expression ratios normalized to the mean of two reference primers (*Gapdh* and *Ywhaz*). Primer sequences are given in Table 1.

CEC culture

Monolayer cultures of primary CECs were cultured as described previously[24]. After 24 hours, the cells were incubated with *T. muris* excretory/secretory antigen 50μg/ml, *T. muris* eggs (10 eggs/well) or medium for 24 hours. The supernatant was then harvested and analysed by ELISA.

Bone marrow dendritic cell culture

Bone marrow was harvested and cultured as previously described[23]. On day six of the culture the cells were harvested from the plates and semi-adherent cells removed. The cells were spun at 400g and re-suspended at a concentration of 1×10^6 cells/ml in DC medium (RPMI 1640 supplemented with 10% LPS-free FBS (Gibco, Paisley, UK) 1% Penicillin/ streptomycin and 50mM Beta-mercaptoethanol (Sigma Aldrich, Dorset UK)). The cells

were stimulated overnight with LPS (100ng/ml) or *T. muris* antigen (5μg/ml). After 24 hours the cells were harvested and prepared for flow cytometry. FcR were blocked by incubating cells in anti-CD16/32 (2μg/ml) for 15 minutes. Cells were washed and stained with anti-CD45 PeCy7 (1 μg/ml, Becton Dickinson), anti-CD11c Alexa700 (1μg/ml), anti-MHC-II FITC (2.5 μg/ml) and anti-CD86 PE (1 μg/ml) (all E Bioscience) and acquired using a LSRII flow cytometer (Becton Dickinson Biosciences, Oxfordshire). Data was analysed using Flow Jo flow cytometry analysis software (Tree Star, Inc, Oregon, US).

Chemokine migration assay

Colonic lamina propria cells from wild type or *Nod2*$^{-/-}$ mice were labelled with Vybrant fluorescent dye (Molecular Probes, Leiden, The Netherlands) and added to the upper well of transwell plates (Fisher Scientific, Loughborough, UK) at 1-8×10^5 per well and chemokines were added to the bottom well (CCL2 (10ng/ml) and CCL2 (1ng/ml), both R and D Systems, Abingdon, UK). As a control, cells were also plated in the absence of chemokine. After incubating for 1h at 37°C cells in the bottom well were stained with CD11c antibodies and the total number of dye-labelled cells counted using an Olympus BX51 upright microscope, captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices).

Statistics

Statistical analyses were performed using a students T test, tow way anova or a Kruskal Wallis test with a Dunn's multiple comparison test. P-values < 0·05 were considered significant. All statistical analyses were carried out using GraphPad Prism for windows, version 3·02.

Results

Up-regulation of Nod2 expression by CEC in response to *T. muris* infection

Trichuris muris penetrates the colonic epithelial layer with its head remaining buried within the epithelium for the bulk of its life cycle. Therefore, we investigated the up-regulation of Nod2 and Rip2 in CECs in early *T. muris* infection in C57BL/6 wild type mice by qPCR. Modulation of *Nod2* expression by the parasite was seen within 24 h of infection, with approximately a two fold increase in the expression of *Nod2* in CECs of C57BL/6 mice ($p<0.001$, Figure 1A) at 24 hours post-infection. Expression of *Rip2* was also increased approximately twofold in CECs at 24 hours post-infection (Figure 1B).

Nod2$^{-/-}$ mice have delayed recruitment of CD103$^+$ DCs to the large-intestinal epithelium

Rapid DC recruitment to the colon has been shown to be associated with resistance to *T. muris*[7]. To assess whether Nod2 plays a role in recruitment of DCs in *T. muris* infection we analysed the recruitment of macrophages and DCs to the large intestine in response to *T. muris* infection (Figure 1C-E). There was no difference between the proportion of CD103$^+$ DCs (CD11c$^+$ MHCII^{hi}) DCs in the large intestine of naïve C57BL/6 WT and *Nod2*$^{-/-}$ animals (Figure 1D). At D1 post infection we observed an increase in the proportion of CD103$^+$ DCs (CD11c$^+$ MHCII^{hi}) recruited to the large intestine in C57BL/6 WT animals but not in *Nod2*$^{-/-}$ mice (Figure 1D, P=0.02). Although proportions of CD103$^+$ DCs

between C57BL/6 WT and *Nod2*$^{-/-}$ mice were similar at D2 the difference in magnitude of the DC response between *Nod2*$^{-/-}$ and C57BL/6 WT mice was more dramatic at D5 post infection, (p=0.01), with C57BL/6 mice showing a 3-fold increase in the proportion of large-intestinal DCs (~17% of the CD45^{+} population) compared to *Nod2*$^{-/-}$ animals in which there was only a modest increase (~5% of the CD45^{+} population). The percentage of DCs remained higher in C57BL/6 mice at Days 7 and 9 post-infection (Figure 1D) compared with *Nod2*$^{-/-}$ mice. We observed no difference in the intensity of expression of CD103 suggesting there was no up-regulation of CD103 expression post-infection (data not shown). In contrast, to recruitment of CD103+ DCs no difference was observed in the proportion of CD103^{-} negative colonic DC populations between *Nod2*$^{-/-}$ and C57BL/6 WT at all time points analysed (D1 *Nod2*$^{-/-}$ 2.00% ±0.88, C56BL/6 3.72% ±1.5, D2 *Nod2*$^{-/-}$ 1.37% ±0.08, C56BL/6 1.45% ±0.78, D5 *Nod2*$^{-/-}$ 7.64% ±1.25, C57BL/6 6.5% ±1.6 D7-D9, data not shown). Overall, the magnitude of CD103^{+} DC recruitment in *Nod2*$^{-/-}$ animals in response to infection was reduced at all time points studied.

To see if there was a general reduction in the phagocyte response to *T. muris in Nod2*$^{-/-}$ mice, we also assessed macrophages (F4/80^{+}MHCII^{+}) (Figure 1E). In contrast to the increased proportions of DCs in WT but not *Nod2*$^{-/-}$ mice post-infection, there was no difference in the proportion of large-intestinal macrophages between *Nod2*$^{-/-}$ and C57BL/6 mice before or after infection at all timepoints studied (Figure 1E). Interestingly, the peak of DC recruitment at D5 post-infection seen in WT mice also corresponded to a small peak in macrophage recruitment although there was no difference between WT and *Nod2*$^{-/-}$ mice with both strains having an equivalent response. To see if the changes in the proportions of DCs/macrophages post-infection between WT and *Nod2*$^{-/-}$ mice were restricted to the large intestine, we also investigated macrophages and DCs proportions in the MLN and spleen and saw no differences.

Immunohistochemistry analysis was used to validate the flow cytometry findings and define phagocyte localisation pre-and post-infection. Results showed higher frequencies of CD11c^{+} cells in the colon and caecum of C57BL/6 mice compared with *Nod2*$^{-/-}$ mice (Figure 2 A and B). As CD11c alone is not a discrete marker of DCs, we performed further validation using antibodies against CD103, CD11b or F4/80 and counted the number of CD103^{+} CD11c^{+} CD11b^{+}cells and F480^{+}CD11c^{+} cells (Figure 2 C-F). Our data showed an increase in the number of CD11c^{+}CD103^{+} cells in C57BL/6 WT mice (Figure 2C and D, p=0.04) and verified the reduced number of CD103^{+}CD11c^{+} DCs in the large intestine of *Nod2*$^{-/-}$ animals (Figure 2). The majority of DCs in both WT and *Nod2*$^{-/-}$ animals were CD11b^{+} in early infection with no significant differences in the proportions of CD11b^{+} DC between strains or post-infection (83.%±15.8 in naïve WT mice versus 84.29±7.37% in naïve *Nod2*$^{-/-}$ mice; 87.5±7.98% in WT versus 95±5% in *Nod2*$^{-/-}$ mice at Day 1 post-infection; 96.67±28% in WT mice versus 91.89±8.1% in *Nod2*$^{-/-}$ mice at Day 5 post-infection, Figure s1 and data not shown). DC localisation changed during *T. muris* infection. Within the large intestine DCs are scarce, located deep within the lamina propria far away from the epithelium (Figure 2A). Upon *T. muris* infection within the wild-type mice, DCs were observed higher up the crypt axis close or adjacent to epithelial cells whereas this was not observed in *Nod2*$^{-/-}$ mice (Figure 2). The number of F480^{+}CD11c^{+} cells were more variable

post-infection but overall showed little difference in number or distribution between *Nod2*$^{-/-}$ and WT mice with most macrophages restricted to the lamina propria below the crypts (Figure 2E and F).

Increased CD103^{+} numbers is due to migration not proliferation

To assess whether the increased proportion of CD103^{+} DCs observed in the colon of C57BL/6 WT mice was due to *in situ* proliferation or migration of the cells to the site of infection we assessed BrdU uptake in DCs in the colon of *Nod2*$^{-/-}$ and C57BL/6 WT animals post-infection by flow cytometry. At D5 p.i., low levels of CD103^{+} BrdU^{+} dual positive cells were observed in C57BL/6 and *Nod2*$^{-/-}$ animals at equivalent levels (Figure 1 F). This data indicates that the increased proportion of DCs observed in C57BL/6 wild type mice compared with *Nod2*$^{-/-}$ is not due to *in situ* proliferation and more likely due to altered DC recruitment into the large intestine.

Impaired *T. muris* expulsion kinetics in *Nod2*$^{-/-}$ mice

To assess whether the delayed recruitment of CD103^{+} DCs impacted on parasite expulsion, *Nod2*$^{-/-}$ and C57BL/6 mice were infected with *T. muris* and worm burdens assessed at D21. C57BL/6 mice are resistant to infection and most mice expel their worms by day 21 post-infection. However, whereas most of the C57Bl/6 mice had expelled their worms, *Nod2*$^{-/-}$ animals had not and there was a significantly higher worm burden in *Nod2*$^{-/-}$ animals compared with C57BL/6 WT mice (Figure 3A, p=0.006). In addition, we measured IFN-γ and IL-13 levels from mesenteric lymph node cells re-stimulated with *T. muris* E/S antigens at day 21 post-infection the peak cytokine response. Both strains of mice produced IFN-γ and IL-13, which is typical of the mixed Th1/Th2 response observed in C57BL/6 mice[2]. Levels of IFN-γ and IL-13 were similar between *Nod2*$^{-/-}$ and C57BL/6 WT mice (Figure 3B and 3C). In addition, we observed no difference in the levels of IL-4, IL-6, IL-9, tumour necrosis factor α, and IL-12p70 observed in *Nod2*$^{-/-}$ and C57BL/6 mice. However, as both strains of mice had expelled some worms by D21 post-infection we looked at the early T cell response (D7, 10 and 18 post-infection) during *T. muris* infection in *Nod2*$^{-/-}$ mice to see if the early dynamics of the T cell response were altered. Analysis of T cells in the MLN revealed a reduction in both CD4 and CD8 T cells in *Nod2*$^{-/-}$ mice early post-infection (Figure 3D and E). We then further analysed T cells positive for the gut homing marker CCR9 and saw that both CD4^{+} CCR9^{+} (Figure F) and activated/ memory cells CD44^{hi} CD62L^{lo} (Figure G) were significantly reduced at day 18 in the MLN of *Nod2*$^{-/-}$ mice. Collectively this data suggests that the reduced DC response in *Nod2*$^{-/-}$ mice impairs the early adaptive immune response to *T. muris*.

Nod2 does not drive basophil recruitment

Basophils have been implicated in mediating Th2 immunity against *T. muris*[25]. Furthermore DCs alone were shown to be insufficient at mediating immunity to *T. muris*[25]. We therefore assessed basophil frequencies in C57BL/6 and *Nod2*$^{-/-}$ post infection with *T. muris* (Figure 4). At D5 post infection there was no difference in the frequency of basophils in C57BL/6 and *Nod2*$^{-/-}$ mice, suggesting no role for Nod2 in the recruitment of basophils in *T. muris* infection (Figure 4 A and B).

Nod2$^{-/-}$ DCs can migrate normally *in vivo*

Nod2 is expressed by intestinal epithelial cells and immune cells therefore we addressed whether the impaired recruitment of DCs was due to an inability of *Nod2*$^{-/-}$ DCs to migrate to the epithelium or due to impaired responses by *Nod2*$^{-/-}$ epithelial cells. Chemokines are key drivers in the migration of immune cells to sites of infection. We therefore first assessed the level of expression of the chemokine receptors CCR5 and CCR2 on DCs by flow cytometry in naive and infected mice and showed that the levels of chemokine receptors were equivalent (Figure 5A and B). These data imply that *Nod2*$^{-/-}$ DCs have a similar potential and ability to respond to chemokines as WT DCs. Indeed, we performed further analysis of DC migratory function by *in vitro* chemotaxis assay (Figure 5C). Our data shows that equivalent numbers of colonic DCs from *Nod2*$^{-/-}$ and C57BL/6 were able to migrate towards CCL5 and CCL2. In the event that there could be a defective response of *Nod2*$^{-/-}$ DCs to parasite, we also investigated the response of DCs to *T. muris* antigen. *T. muris* reside within an epithelial syncitial tunnel and does not breach the epithelial layer, therefore, we investigated the interaction of DCs with parasite secretory antigen which may cross the epithelial barrier. Our data shows *Nod2*$^{-/-}$ DCs up-regulated MHC-II (Figure 5 D), CD86 and CD80 (data not shown) at equivalent levels to C57BL/6 WT animals in response to LPS or *T. muris* antigen. Combined, these data suggest that *Nod2*$^{-/-}$ DC migratory function and ability to respond to *T. muris* is normal.

We next assessed the ability of *Nod2*$^{-/-}$ DCs to migrate to *Nod2*$^{+/+}$ epithelium *in vivo* by generating bone marrow chimeras. C57BL/6 WT mice irradiated and reconstituted with *Nod2*$^{-/-}$ bone marrow (herein referred to as C57BL/6$^{nod2-/-}$ (Figure 6A) were used to assess the responses of Nod2 deficient DCs to Nod2 sufficient CECs in response to *T. muris* infection. Successful reconstitution of C57BL/6 wild type with *Nod2*$^{-/-}$ bone marrow was assessed by determining Nod2 ligand responsiveness of isolated DCs (Figure 6B). As expected bone marrow DCs derived from C57BL/6$^{nod2-/-}$ were unresponsive to the Nod2 ligand (p=0.01), MDP, but were responsive to LPS, identifying the bone marrow cells as being of *Nod2*$^{-/-}$ origin and that chimerism had been achieved. Colonic cells from the chimeras at D5 post infection were assessed for the presence of CD103^{+} DCs. In C57BL/6$^{nod2-/-}$ CD103^{+} DCs had migrated to the colonic epithelium with the proportion of DC being equivalent to that of normal C57BL/6 mice (Figure 6C). We did attempt to perform a reverse bone marrow chimera and reconstituted *Nod2*$^{-/-}$ mice with WT bone marrow (Nod2^{wt}). When we irradiated Nod2 deficient mice, the majority of the mice died within a few days post-irradiation which we attributed to a defect in epithelial homeostasis that we published previously[14]. However, of the mice that survived we were able to confirm a defect in immune cell recruitment to *T. muris* infection (Figure 6D, p=0.01) and DC recruitment with only 7.1±1.1 CD103^{+}CD11c^{+} DCs in the lamina propria of Nod2^{wt} chimeras compared with 17.75±3.2 DCs in WT^{nod2} chimeras (Figure 6E, p=0.01). Collectively this data demonstrates that *Nod2*$^{-/-}$ DCs are able to migrate effectively *in vivo* in response to *T. muris*.

Nod2$^{-/-}$ epithelial cells have impaired chemokine secretion

Previous data from our group showed a role for epithelial cell-derived chemokines in DC recruitment to *T. muris*[7]. It is possible therefore that the impaired recruitment of CD103^{+}

DCs in *Nod2*$^{-/-}$ mice was due to defective epithelial responses and production of chemokines. To assess CEC function in *Nod2*$^{-/-}$ mice, CECs were harvested from the epithelium of C57BL/6 wild type and *Nod2*$^{-/-}$ mice and cultured *in vitro* with *T. muris* E/S antigen, *T. muris* eggs and a medium control using our validated isolation and culture methods and the supernatants analysed by ELISA (Figure 7 and data not shown). Cultured CECs from C57BL/6 WT animals constitutively produced CCL2, CCL5 and CCL20 (Figure 7A-D). In contrast, CECs from *Nod2*$^{-/-}$ mice produced significantly lower amounts of CCL2 ($p<0.001$) and CCL5 ($p<0.05$, Figure 7 A and B). There was no significant reduction in CCL20 secretion from *Nod2*$^{-/-}$ CECs compared with WT CECs. (Figure 7C). Importantly *Nod2*$^{-/-}$ CECs were able to secrete chemokines albeit at a reduced magnitude compared with WT CECs. Indeed, levels of secretion of the alarmin IL-33 showed that secretion was equivalent in WT and *Nod2*$^{-/-}$ CECs (Figure 7D). Addition of E/S antigen or *T. muris* eggs did not increase chemokine secretion from C57BL/6 WT *or Nod2*$^{-/-}$ CEC (data not shown) suggesting that epithelial cells were not responding to either eggs or parasite secretory antigen. As we saw a significant difference in the levels of CCL3 and CCL5 by ELISA we also investigated levels of these chemokines in epithelial cells *in vivo* by qPCR. qPCR data showed a trend for reduced expression of *CCL5*, *CCl2* and *IL33 in Nod2*$^{-/-}$ CEC post-infection although this was not significant (Figure 7E-G).

Discussion

The immune response to *T. muris* is well characterised, with resistance being associated with an archetypal Th2 response and susceptibility with a dominant Th1 response[26]. However, the mechanisms that govern the initiation of the immune response, in terms of the recognition of infection, and recruitment of immune cells to the site of infection are unclear. The findings from our study document a role for Nod2 in CEC in the production of chemokines that drive CD103^{+} DC recruitment the epithelium in response to *T. muris* infection. These findings shed new light on the role of PRRs and Nod2 in initiating host responses to invading pathogens in the colon.

Nod2 has been previously shown to be important in the production of chemokines. Consistent with our findings, a recent study showed that *Nod2*$^{-/-}$ mice had impaired secretion of CCL2 from the stromal compartment of the small intestine in response to bacterial infection[27]. Furthermore, as we have seen here, this impaired chemokine production impacted on the recruitment of inflammatory monocytes to the site of infection[27]. Inflammatory monocytes have been shown to differentiate into DCs[28, 29] and macrophages[30] with the differentiation pathway of monocytes depending on the type of environmental and the nature of the infection[31]. This prior study did not include an analysis of CD103^{+} DCs or a determination of whether the recruited monocytes differentiated into DCs or macrophages. Furthermore, this prior study also showed that epithelial cells infected with *Citrobacter rodentium* did not produce CCL2 and that CCL2 production is unique to stromal cells beneath the epithelium. However, this was determined using an epithelial cell line rather than primary epithelial cells. We were able to see epithelial derived CCL2 by qPCR and ELISA suggesting differences may exist between primary cells and cell lines. The purity of CECs used in our study was >98%[24] making it highly unlikely that the CCL2 production detected was due to stromal cell contamination.

Furthermore, previous work from our group has shown that epithelial-derived chemokine secretion is important in driving the recruitment of DCs to the large intestine[7] with the chemokines CCL5 and CCL20 particularly implicated. We observed lower levels of detectable chemokines from WT CECs in our model compared to our previously published data. In the previous study, only BALB/c and AKR mice were analysed and not C57BL/6 mice. Thus there may be differences in the different mouse strains particularly when you consider that unlike BALB/c or AKR mice which have well defined and polarised responses to *T. muris*, C57BL/6 mice have a mixed Th1/Th2 response. Furthermore, it is not clear that CCL5 is critical for DC recruitment in resistant BALB/c mice as although blocking both CCL5 and CCL20 prevented DC recruitment, CCL5 was not tested alone. Other epithelial derived chemokines may also be important in DC recruitment and it should be noted that our previous studies investigated CCL5, CCL20, CCL3 and CCL2[7] all of which were reduced in susceptible mice.

The defect in immune cell recruitment seen in *Nod2*$^{-/-}$ mice was specific to CD103^{+}CD11c^{+}MHC^{high} DCs and not basophils or macrophages. When looking at the MHCII^{low} population all cells were F4/80 negative. CD103+ MHCII^{low} frequencies were low but were the same between *Nod2*$^{-/-}$ and C57BL/6 mice. In addition, we looked at CD11b expression by flow cytometry and immunohistochemistry and found it to be expressed on macrophages and DCs, and despite their being a small population of CD103^{+} CD11b^{+} cells in the large intestine we did not observe differences in the frequency of these cells between *Nod2*$^{-/-}$ and C57BL/6 mice in early infection. This suggests Nod2 has an important role in CD103^{+} DC recruitment in the response to *T. muris.* Indeed, although macrophages have been shown to be recruited to the large intestine in *T. muris* infection this is much later in infection (D21 PI) [32] suggesting they play different roles in orchestrating immunity to *T. muris* and other mechanisms aside from Nod2 activation in the epithelium drive their recruitment or proliferation. DC migration to the site of infection is an important stage in the immune response[33], with many studies showing that DCs are necessary for priming adaptive immunity. For example, effective DC migration is needed to prevent susceptibility to infection with the protozoan parasite, *Cryptosporidium parvum*[34]. Therefore, due to the observed impaired DC recruitment we expected *Nod2*$^{-/-}$ mice to be susceptible to *T. muris* infection. Indeed, analysis of worm burdens at D21 post infection showed that *Nod2*$^{-/-}$ mice had delayed expulsion of *T. muris.* Furthermore, analysis of early T cell responses at D7, 10 and 18 post-infection demonstrated that whereas there was a clear response and expansion of T cells in the MLNs of WT mice at D18 post infection, this was significantly reduced in *Nod2*$^{-/-}$ mice. Strikingly, when we looked at the numbers of T cells positive for the gut homing receptor CCR9 we observed a significantly lower number in *Nod2*$^{-/-}$ animals. This observation could explain why we see impaired parasite expulsion at D21 post infection. We suggest that impaired DC recruitment to the colon in the early stages of the immune response against *T. muris* then reduces priming of the early adaptive T cell immune response in the MLN. Thus, less T cells are primed and initiated to migrate to the gut which impacts on the initial stages of parasite expulsion. However, this deficit in DC and T cell numbers is overcome as mice are ultimately able to expel infection suggesting that the reduced DC responses alters the dynamics of the immune response rather than the overall outcome to infection.

We show, via an *in vitro* chemotaxis assay, that dendritic cells from both *Nod2*$^{-/-}$ and C57BL/6 mice are able to migrate effectively towards a chemotactic stimulus suggesting that *Nod2*$^{-/-}$ DCs are functional, at least in their migratory capabilities. This, coupled with our data from the generation of bone marrow chimeras allows us to propose that it is Nod2-dependent epithelial responses that promote DC recruitment to the large intestinal epithelium. This then could impact on the downstream immune response such that there is a delayed expulsion of the parasite. Furthermore, whilst DC numbers did increase at D2 and D5 post infection in the *Nod2*$^{-/-}$ mice the magnitude was not as great as that observed in WT mice. Thus, DC migration is both reduced and delayed in *Nod2*$^{-/-}$ mice. However, the small level of DC migration in the *Nod2*$^{-/-}$ mouse suggests that other mechanisms other than epithelial Nod2 signalling are involved in DC recruitment to the large intestine. For example, eosinophils have been shown to be important for DC recruitment to the lymph nodes that drain the lungs[35]. As eosinophilia is also observed during *T. muris* infection[36] eosinophils may be involved in DC recruitment.

Importantly, *Nod2*$^{-/-}$ mice were not devoid of DCs and DCs from *Nod2*$^{-/-}$ mice were shown *in vitro* to be capable of responding to *T. muris* antigen thus suggesting that *Nod2*$^{-/-}$ the DCs are still functioning effectively. In addition, as *Nod2*$^{-/-}$ mice are ultimately resistant to infection and able to expel all parasites by day 35 PI it may be that the few DCs recruited to the colon are sufficient to ultimately initiate an effective albeit delayed immunity. Indeed, coupled with delayed expulsion of the worm, *Nod2*$^{-/-}$ mice were able to mount an effective Th2 response and produced the cytokines IL-13 and IL-4 at equivalent levels to that seen in WT animals despite the impaired DC migration. This is not the first time that effective DC function has been shown to not be paramount to helminth resistance. Experiments restricting antigen presentation to DCs during *T. muris* infection did not impede host clearance of the pathogen or the ability of the host to mount Th2 responses[27]. In these experiments the role of antigen presentation and T cell priming was attributed to basophils. We addressed whether, in the absence of an effective DC recruitment, there was a compensatory recruitment of basophils to the gut. However, our data demonstrated that basophils were detected at equivalent levels in the large intestine of both WT and *Nod2*$^{-/-}$ mice post-infection albeit in very low numbers. Previous data has shown a role for basophils in augmenting Th2 responses[37, 38] therefore basophils may be acting to support the few CD103^{+} DCs observed in the *Nod2*$^{-/-}$ mice in promoting T cell polarisation.

The factors that drive the recognition of *T. muris* infection by Nod2 are still unknown but there are several possibilities. PRRs have been shown to respond to damage associated molecular patterns such as heat shock proteins, ATP and heparin[8]. *T. muris* physically invades the gastrointestinal epithelial through the aid of the secretion of pore forming antigens[39, 40]. During this process the epithelium is put under a lot of stress potentially causing the release of DAMPs that may be detected by Nod2. Indeed, the NLR family member NLRP3 is known to detect DAMPs and cellular stress[41, 42] and previous work has shown synergism between NLRP3 and Nod2[43]. Moreover, *T. muris* infection has been associated with increased apoptosis in susceptible mice[44] and E/S antigen from the porcine parasite *Trichuris suis*, has been shown to have cytotoxic effects on intestinal epithelial cells[45]. Combined, these data support our hypothesis that damage caused during

infection may trigger Nod2 activation. *T. muris* also produces an excretory/secretory antigen (ES antigen) which may contain components that have the potential to bind to Nod2, however, since our data from the CEC cultures failed to show any additional chemokine response to *T. muris* antigen makes this is an unlikely hypothesis. Another possibility is that Nod2 directly recognises surface proteins on the helminth. Currently, we don't know what surface proteins are expressed on *T. muris* and if/how they interact with the epithelium or immune cells. It is not possible to maintain both *T. muris* larvae and viable CEC together *in vitro*, therefore these experiments cannot be performed and thus this possibility cannot be ruled out yet. Finally, as the *T. muris* enters the epithelium, it may allow introduction of bacteria into the epithelial cells, either via opportunistic translocation of bacteria as pores are formed within the epithelial layer, or *T. muris* could directly carry bacteria on its surface into the epithelium, thus triggering PRR engagement.

In conclusion we have provided evidence that shows, for the first time, a role for a PRR in mediating the immune response to the pathogen *T. muris*. Activation of Nod2 mediates the production of epithelial-derived chemokines, which then induces the recruitment of $CD103^+$ dendritic cells to the gastrointestinal epithelium. As DC recruitment to the epithelium has previously been shown to be important for host resistance to *T. muris* infection[7], activation of Nod2 is therefore potentially an important first step in the initiation of the immune response to infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the Biotechnology and Biological Sciences Research Council, UK (SRC), Epistem Ltd. RKG was supported by the Wellcome Trust grant number 083620/Z/07/Z. A special thanks to Dr Kaye Williams and Epistem for their help with the generation of bone marrow chimeras.

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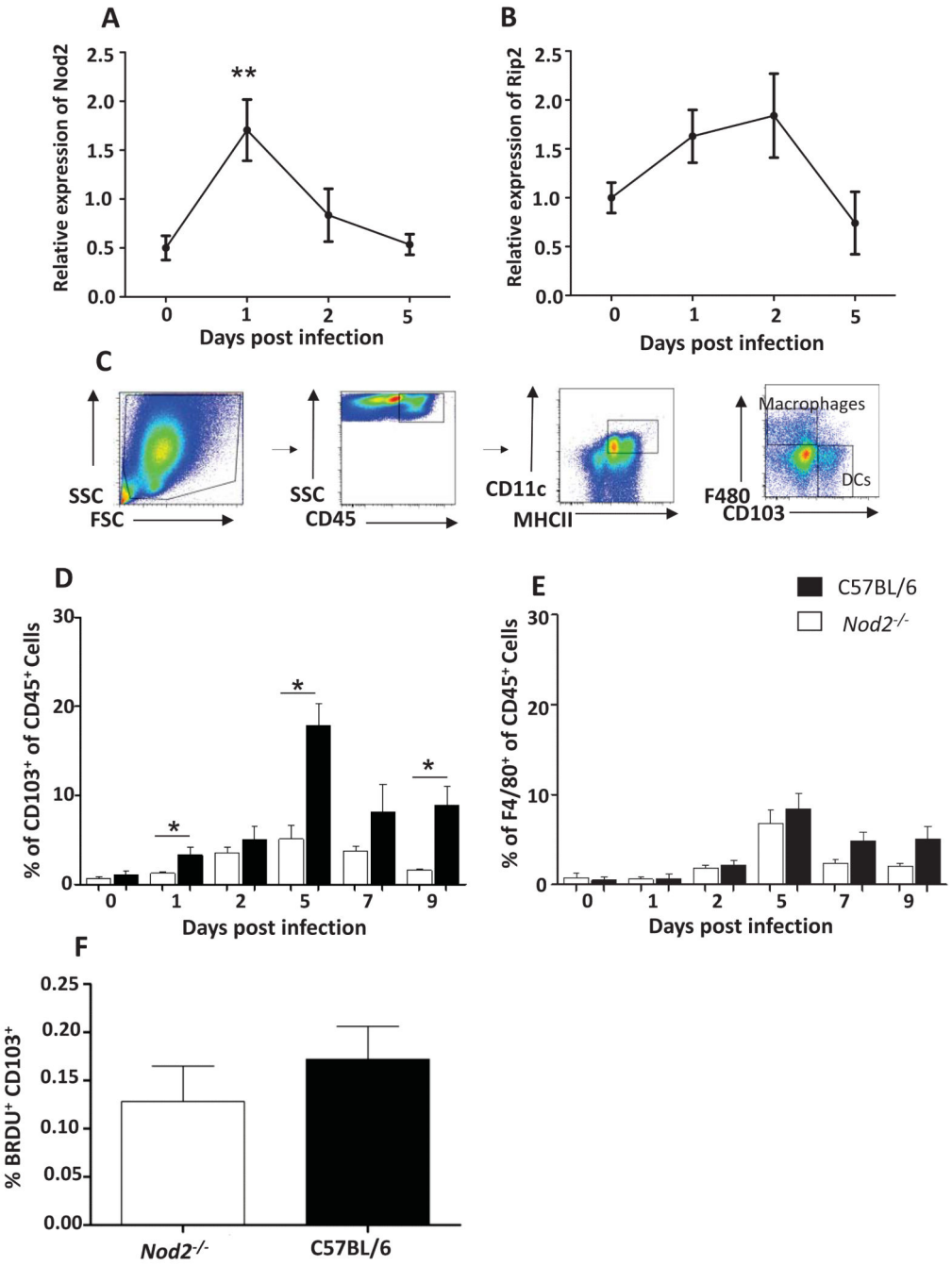

Figure 1. Impaired recruitment of CD103$^+$ DCs to the colonic epithelium in *Nod2*$^{-/-}$ mice in response to *T. muris*

WT and *Nod2*$^{-/-}$ mice were infected orally with approximately 175 embryonated *T. muris* eggs. Colonic epithelial cells from WT mice were analysed before and after infection by qPCR for *Nod2* **(A)** and *Rip2* **(B)** mRNA *n*=3-12 Data for time points D0 and D1 are pooled from two individual experiments. Lamina propria and intraepithelial cells were isolated from the large intestine and stained for CD45, MHCII, CD11c, CD103 and F4/80 on D0, D1, D2, D5, D7 and D9 post infection. (**C**) Gating strategy for CD103$^+$ and F4/80 positive cells

isolated from the large intestine. (**D**) Percentage of MHCII^{+}CD11c^{+}CD103^{+}F4/80^{-} DCs were calculated as a percentage of the CD45^{+} cell population, (**E**) Percentage of MHCII +CD11c+CD103^{-} F4/80^{+} macrophages were calculated as a percentage of the CD45^{+} cell population. Data is representative of at least 3-4 mice each from 3 experiments (total *n*=9-12) with the exception of D7 and D9 which are representative of 1 experiment (*n*=4). (**F**)Mice infected with *T. muris* were injected with BRDU 16 hours before sacrifice. Lamina propria and intraepithelial cells were isolated from the large intestine and stained for CD45, MHCII, CD11c, CD103 and BrdU and the number of BRDU^{+}CD103^{+} of CD45^{+} population quantified.*n*= 5 **P= <0.05*, ***P<0.001*.

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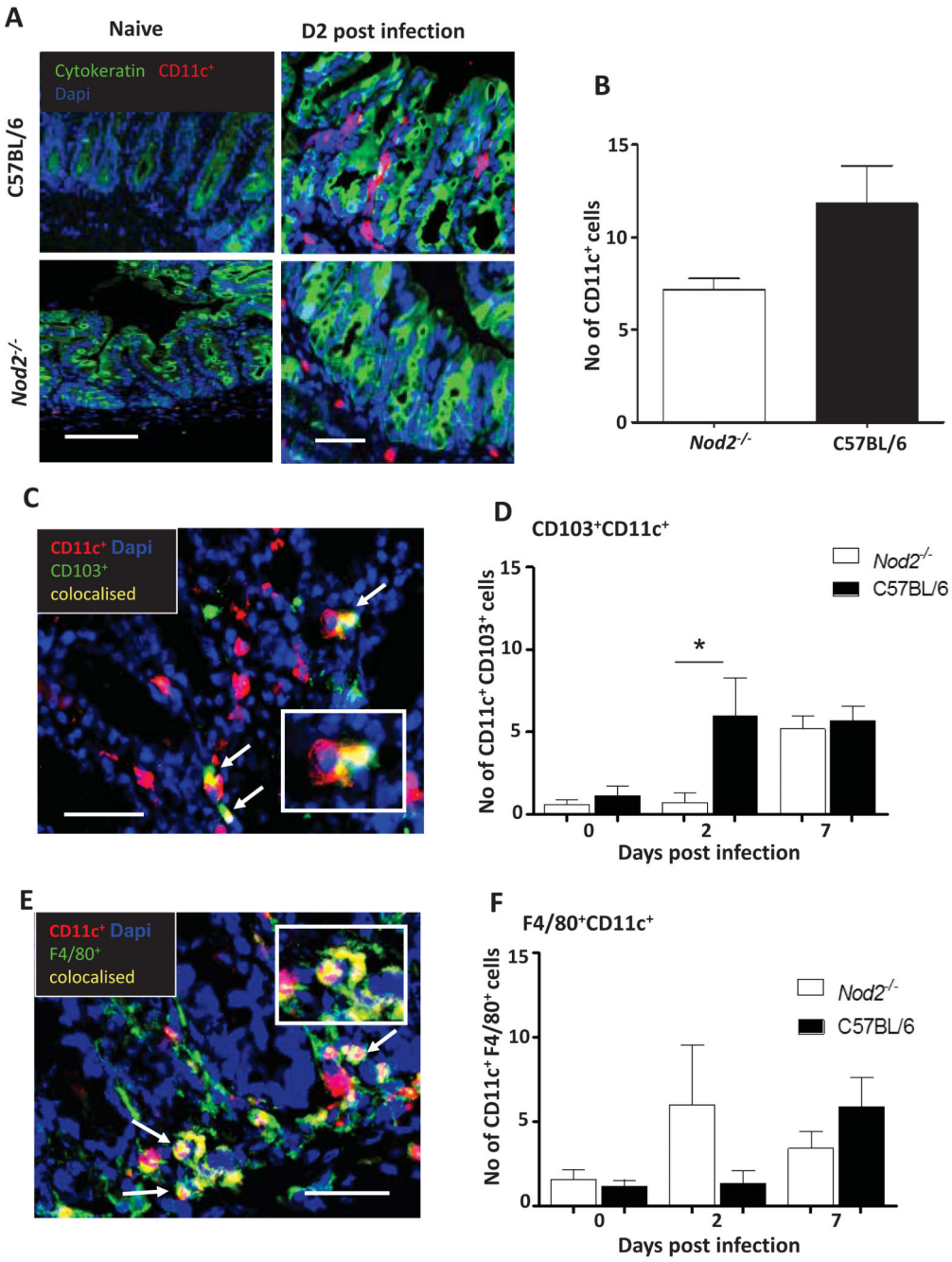

Figure 2. Impaired recruitment of CD103$^+$ DCs to the colonic epithelium in *Nod2*$^{-/-}$ mice in response to *T. muris*

Frozen caecal and colon sections were taken at autopsy, sectioned and stained for nuclei (blue), cytokeratin (green) and CD11c (red). Representative images shown in **A** (Scale bar for naïves = 100μm. D2 p.i. = 50 μm). (**B**) Quantification of CD11c$^+$ cells in *Nod2*$^{-/-}$ and C57BL/6 mice. Frozen caecal and colon sections were taken at autopsy, sectioned and stained for nuclei (Dapi, blue) CD11c (red) and CD103 or F4/80 (green). Co-localised cells are shown in yellow. (**C**) Representative image of CD103 staining. (Scale bar = 50μm

(insert= 30 μm)). (**D**) Quantification of dual stained $CD103^+$ $CD11c^+$ cells. (**E**)Representative image of F4/80 staining. (Scale bar = 50μm (insert= 40 μm)). (**F**) Quantification of dual stained F4/80 CD11c cells. *n* = 3 (C57BL/6) *n*=4 ($Nod2^{-/-}$)/ timepoint. Data shown are mean +/-SEM.

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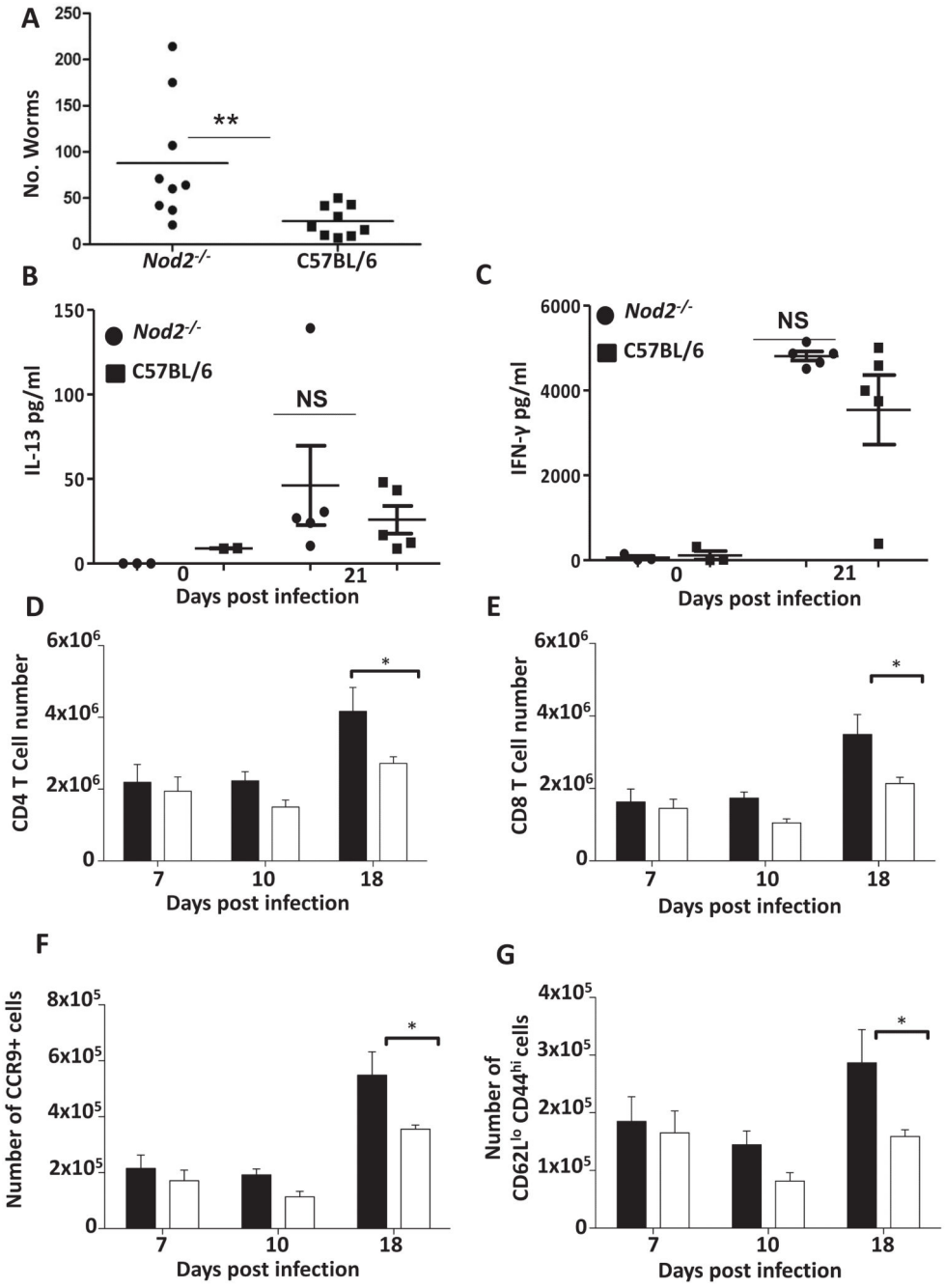

Figure 3. Delayed expulsion kinetics of *T. muris* in *Nod2*$^{-/-}$ mice

Mice were infected orally with approximately 175 embryonated *T. muris* eggs and worm burdens were assessed at D21 post infection (**A**). Data shown are for individual mice with mean values per group. Data is representative of two independent experiments with *n=4* (*Nod2*$^{-/-}$) and *n*=5 (C57BL/6) in each experiment. (**B**) Mesenteric lymph nodes cells re-stimulated with E/S at day 21p.i. IL-13 (**B**) and IFN-γ (**C**) levels in supernatants were then assayed by cytokine bead array. MLN cells were harvested from naïve mice and at D7, 10 and 18 post-infection and the numbers of T cells enumerated (**D** and **E**) and the number of

gut homing (CCR9+) CD4 T cells (F) and activated/memory CD62L^{lo}CD44^{hi} cells (**G**) enumerated by flow cytometry. Analysis of Data is representative of two independent experiments each with *n*=2-4 (naïve animals) and *n*=5 (infected animals). *= *P<0.05*

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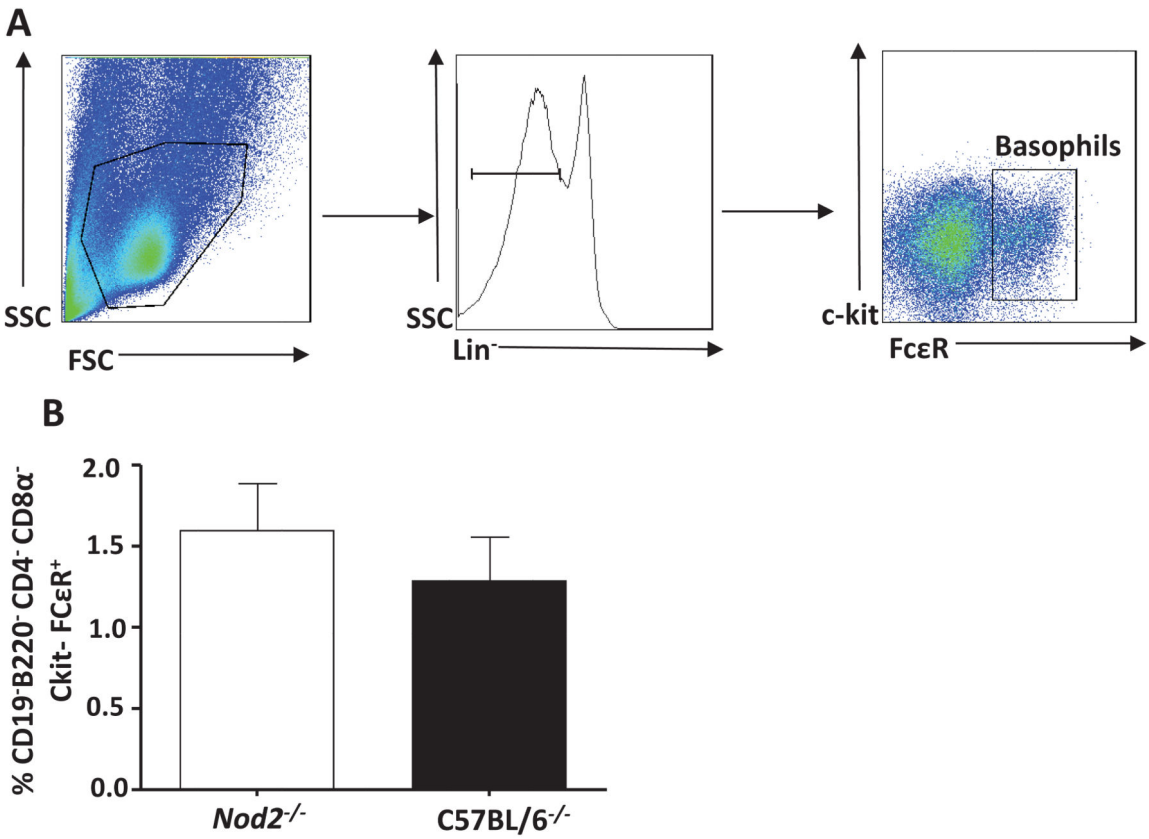

Figure 4. Increased CD103$^+$ cells in the large intestine is not due to *in situ* proliferation of DCs and Nod2 has no role in basophil recruitment to the large intestine

Mice were infected orally with approximately 175 embryonated *T. muris* eggs. Lamina propria and intraepithelial cells were isolated from the large intestine and stained for Lineage markers (CD4, CD8α, B220, CD19), c-kit and FcεR. (**A**)Gating strategy for basophils (Lin$^-$ C-kit$^-$ FcεR$^+$) cells. (**B**) Quantification of Lin$^-$ c-kit$^-$ FcεR$^+$ cells in the large intestine and MLN. *n*= 5 (C57BL/6) *n*= 4 (*Nod2*$^{-/-}$).

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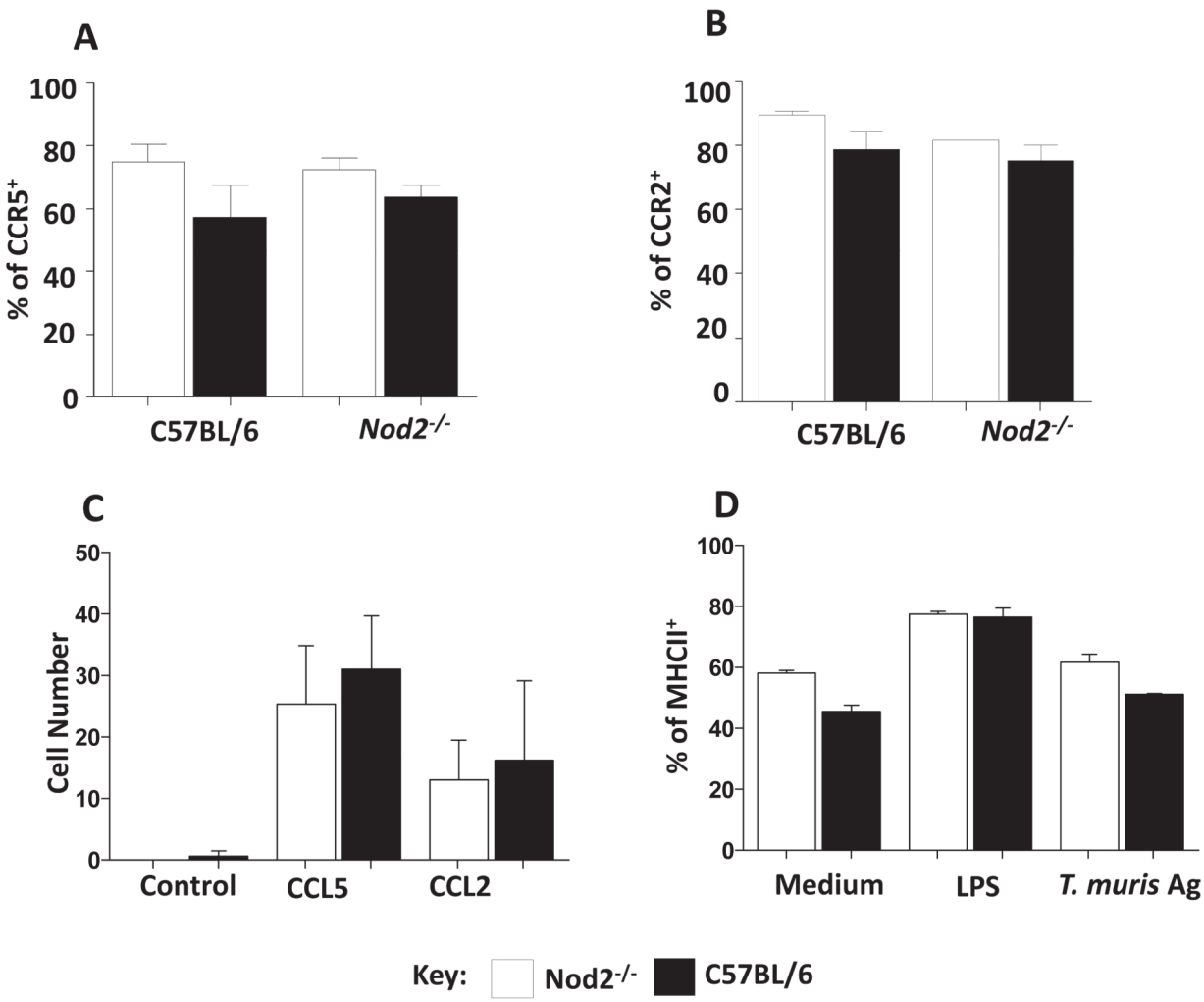

Figure 5. ***Nod2*$^{-/-}$ dendritic cells express chemokine receptors and can migrate effectively *in vitro***

Mice were infected orally with approximately 175 embryonated *T. muris* eggs. Lamina propria and intraepithelial cells were isolated from the large intestine Dendritic cells isolated from the large intestine were stained with chemokine receptors CCR5 **(A)** and CCR2 **(B)**. **(C)** A chemotaxis assay was performed using colonic DCs and CCL2 (10ng/ml) and CCL5 (1ng/ml) *n*= 5 (C57BL/6) *n*= 3-4 (*Nod2*$^{-/-}$). **(D)** BMDC were cultured with medium (negative control), LPS 5μg/ml) or *T. muris* antigen (5μg/ml) for 24 hours before being stained for CD45, CD11c and MHC-II and analysed by flow cytometry. Data shown is the average of three replicates and is representative of two experiments.

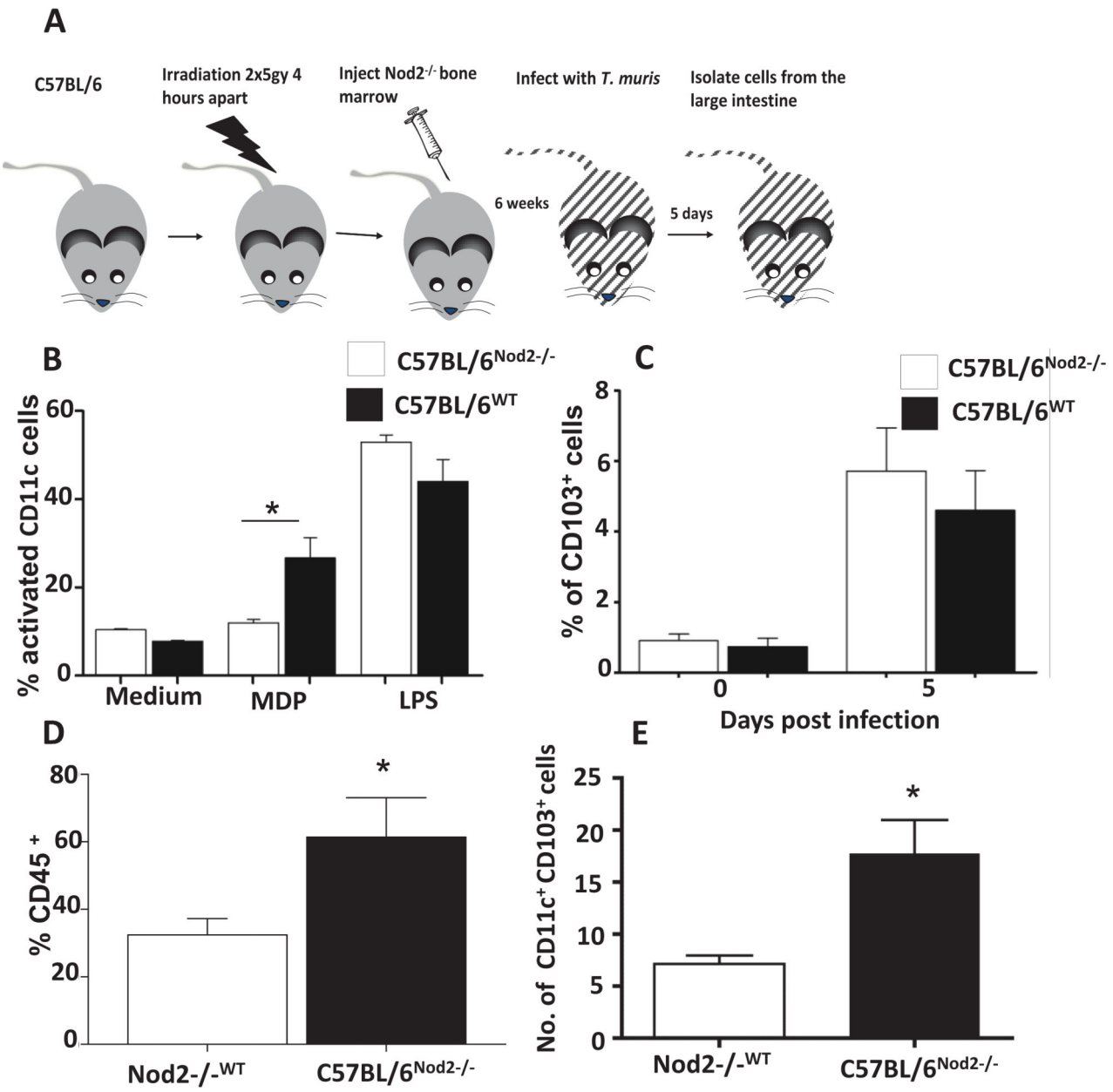

Figure 6. ***Nod2*$^{-/-}$ dendritic cells can migrate to a *Nod2*$^{+/+}$ epithelium**

(A) A schematic representation of generation of bone marrow chimeras. **(B)** Successful reconstitution was determined by harvesting bone marrow from C57BL/6$^{Nod-/-}$ mice and stimulating with medium (negative control), MDP (1.0μg/ml) and LPS (100ng/ml, positive control). DC responses to the various ligands were analysed by assessing the level of maturation by CD86, CD40 and MHC-II up-regulation by flow cytometry. The graphs show the percentage of MHCII^{hi}CD40^{+} CD86^{+} CD11c cells in each culture condition. (**C**) Lamina propria and intraepithelial cells were isolated from the large intestine of C57BL/6$^{Nod-/-}$ and C57BL/6^{wt} mice and stained for CD45, MHC-II, CD11c, CD103 and F4/80 on D5 post infection and measured as a percentage of the CD45^{+} population. (*n*=4-5) **(D)** A defect in

CD45 cell recruitment was also observed in Nod2^{wt} mice compared with controls. **(E)** Immunohistochemistry staining revealed a defect in CD11c^{+}CD103^{+} DC recruitment in Nod2^{wt} mice compared with C57BL/6$^{Nod-/-}$ mice. * p=0.01.

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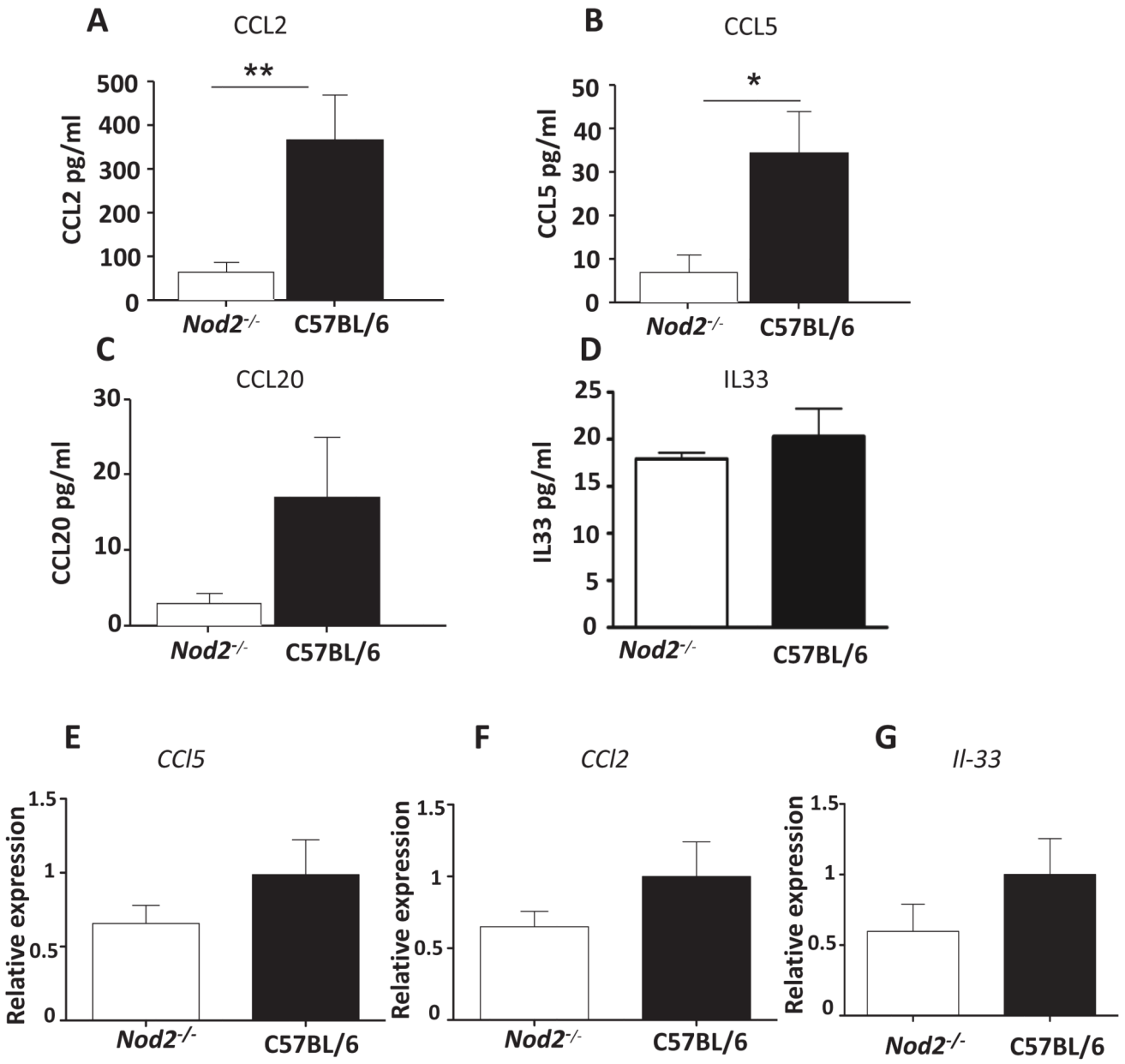

Figure 7. *Nod2*$^{-/-}$ colonic epithelial cells are unable to produce the chemokines CCL2 CCL5 and CCL20

Colonic epithelial cells were cultured from C57BL/6 and *Nod2*$^{-/-}$ mice and the levels of CCL2, CCL5, CCL20 and IL33 were measured by ELISA **(A-D)** (*n*=8 and data is representative of two independent experiments). Colonic epithelial cells were harvested from the colon of naïve and infected C57BL/6 and *Nod2*$^{-/-}$ mice on D1 post infection. mRNA levels of CCL5, CCL2 and IL-33, were measured by qPCR **(E-G)** and data shows relative expression (*n*=11-13 and is pooled from two individual experiments).

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Table 1

Primer sequences used for quantitative PCR of colonic epithelial cells

Gene	Forward Primer	Reverse Primer
Gapdh	CCCACTAACATCAAATGGGG	TCTCCATGGTGGTGAAGACA
Ywhaz	TTCTTGATCCCCAATGCTTC	TTCTTGTCATC ACCAGCAGC
CCL2	TCTGGGCCTGCTGTTCACA	CTGTCACACTGGTCACTCCTA
CCL5	GGGTACCATGAAGATCTCTGCA	TTGGCGGTTCCTT CGAGTGA
IL33	AGACCAGGTGCTACTACGCTAC	CACCATCAG CTTCTTCCCATCC
Nod2	CGACATCTCCCACAGAGTTGTAATCC	GGCACCTGAAGTTGACATTTTGC
Rip2	CTGCACCCGAAGGCGGAACAATCA	GCGCCCATCCACTCTGTATTAGC