Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity

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Abstract

Innate immunity provides the first line of defence against invading pathogens and provides important cues for the development of adaptive immunity. Type-2 immunity – responsible for protective immune responses to helminth parasites1,2 and the underlying cause of the pathogenesis of allergic asthma3,4 – consists of responses dominated by the cardinal type-2 cytokines interleukin (IL)-4, IL-5 and IL-13 (ref. 5). T cells are an important source of these cytokines in adaptive immune responses, but the innate cell sources remain to be comprehensively elucidated. Here, through the use of novel *Il13eGFP* reporter mice, we present the identification and functional characterisation of a new innate type-2 immune effector leukocyte that we have named the nuocyte. Nuocytes expand *in vivo* in response to the type 2-inducing cytokines IL-25 and IL-33, and represent the predominant early source of IL-13 during helminth infection with *Nippostrongylus brasiliensis*. In the combined absence of IL-25 and IL-33 signalling, nuocytes fail to expand, resulting in a severe defect in worm expulsion that is rescued by the adoptive transfer of *in vitro* cultured wildtype, but not IL-13-deficient, nuocytes. Thus, nuocytes represent a critically important innate effector cell in type-2 immunity.

Type-2 immunity evolved to respond to parasitic helminth infections, with type-2 cytokines orchestrating eosinophilia, goblet cell hyperplasia, mucus secretion, and IgE production5-7. These highly complex host responses involve the co-ordination of innate and adaptive immune cell types. Of the defined innate immune cells, basophils, eosinophils and mast cells are known sources of type-2 cytokines, but it is not clear that they are essential for *N. brasiliensis* expulsion5,8-12.

To identify new cell types that may mediate type-2 immunity we investigated the cellular sources of IL-13, a critical cytokine in the host response to helminth infection7,13 and allergy6,14. To allow live imaging of enhanced green fluorescent protein (eGFP) as a surrogate for IL-13 gene expression during the induction of type-2 responses we generated *Il13eGFP* mice (Supplementary Fig. 1). Analysis of these mice revealed very few

constitutive eGFP$^+$ cells in naïve mice (Supplementary Fig. 1). Administration of IL-25 or IL-33 to *Il13eGFP* mice resulted in the detection of ~3% eGFP$^+$ cells in the mesenteric lymph nodes (mLN) (Fig. 1a), at least 80% of which could not be assigned to known cell lineages (including T cells, B cells, natural killer (NK) T cells, natural killer (NK) cells, dendritic cells, neutrophils, eosinophils, mast cells, basophils or macrophages) using a spectrum of cell surface markers (Fig. 1a and b, and Supplementary Fig. 2a). Immunofluorescence revealed highly increased numbers of eGFP$^+$ cells in the intestines (Fig. 1c) and spleens (Supplementary Fig. 2b) of both IL-25 and IL-33-treated *Il13eGFP* mice, and these were confirmed to be non-T cells. The lineage$^−$eGFP$^+$ cells were T1/ST2$^+$ (IL-33R) and IL-17BR$^+$ (IL-25R) (Fig. 1b), suggesting that they respond directly to IL-33 and IL-25. Analysis of *il17br$^{-/-}$* mice (Supplementary Fig. 3) and *Il1rl1$^{-/-}$* mice demonstrated that exogenous IL-25 and IL-33 act redundantly to induce these cells *in vivo* (Fig. 1d). These lineage$^−$eGFP$^+$T1/ST2$^+$IL-17BR$^+$ cells represent a novel IL-13-producing leukocyte population that we have named nuocytes due to their high level expression of IL-13, and with *nu* being the 13th letter of the Greek alphabet. As discussed below nuocytes can additionally be defined as lineage$^−$ cells expressing ICOS, T1/ST2, IL-17BR and IL-7Rα.

Though present in the spleen, mesenteric lymph node and bone marrow of naïve mice, nuocytes represent less than 0.2% of cells in each tissue, but increase significantly in these tissues (Supplementary Fig. 4), with the exception of bone marrow (data not shown), following intra-peritoneal administration of IL-25. In contrast, basophil numbers did not increase in the blood or spleen, and their IL-4 production was unaffected, by IL-25 treatment (data not shown). Confirming that nuocytes were not T or B cells, mast cells, NKT cells or lymphoid tissue inducer (LTi) cells, we detected IL-25-driven nuocyte induction in *Rag2$^{-/-}$* mice and nude mice, *Kit$^{W-sh/W-sh}$* mice, *Ja18$^{-/-}$* mice and *Rorg$^{-/-}$* mice, respectively (Supplementary Fig. 5a-d and data not shown). Furthermore, microarray analysis of highly purified nuocytes failed to show any significant gene expression similarity to known leukocyte lineages (Fig. 1e). However, it did reveal a number of cell surface markers (Supplementary Table 1), including the receptor for IL-7 and the co-stimulatory molecule ICOS and MHC class II, that were subsequently confirmed by flow cytometry as being expressed on nuocytes (Supplementary Fig. 2c).

Importantly, nuocytes represent the predominant cell type expressing *Il13eGFP* at day 5 post-infection (p.i.) with the helminth parasite *N. brasiliensis* (Fig. 2a and Supplementary Fig. 2d). To investigate the potential roles of IL-25 and IL-33 in regulating nuocytes during helminth infection we infected *Il17br$^{-/-}$*, *Il1rl1$^{-/-}$* and combined *Il17br$^{-/-}$Il1rl1$^{-/-}$* mice with *N. brasiliensis*. *Il17br$^{-/-}$* mice expelled their worms more slowly than wildtypes, but by day 14 even *Il17br$^{-/-}$* mice had relatively few worms and a complete absence of worms by day 20 p.i. (Fig. 2b). By contrast, *Il1rl1$^{-/-}$* mice efficiently expelled their worm burden with very few worms present at day 11 and complete absence by day 14 (Fig. 2b). Strikingly, the absence of both IL-25 and IL-33 signalling severely impaired worm expulsion from the *Il17br$^{-/-}$Il1rl1$^{-/-}$* mice, with significant worm burden persisting to day 20 post-infection (Fig. 2b). Using *Il17br$^{-/-}$Il13eGFP* mice and *Il1rl1$^{-/-}$Il13eGFP* mice we found that the loss of either IL-17BR or T1/ST2 resulted in a substantial fall in the numbers of eGFP$^+$ cells early in the response (Fig. 2c). Notably, the expansion of nuocytes in the various mouse strains correlated faithfully with the onset of worm expulsion. Thus, nuocytes arose more rapidly in *Il1rl1$^{-/-}$* mice (though more slowly than in wildtype controls) than in *Il17br$^{-/-}$* mice. Nuocytes failed to expand in the combined *Il17br$^{-/-}$Il1rl1$^{-/-}$* mice in either the MLN (Fig. 2d) or peritoneal lavage (Supplementary Fig. 6a), even at day 20 p.i. Although eosinophils and IgE levels were also reduced in the *Il17br$^{-/-}$* mice, *Il1rl1$^{-/-}$* mice and *Il17br$^{-/-}$Il1rl1$^{-/-}$* mice (Supplementary Fig. 6b and c), these have been shown by others not

to be essential for worm expulsion5,15. In addition, we found no defect in basophil expansion in the *Il17br*$^{-/-}$ mice (data not shown and Supplementary Fig. 7b).

To address the functional importance of nuocytes in the immune response to helminth infection, we purified nuocytes to homogeneity (Fig. 3a) and determined conditions for their expansion *in vitro*. Nuocytes did not grow or differentiate in culture with a cytokine cocktail (CC) used previously to differentiate a basophil/mast cell progenitor *in vitro*16 (Fig. 3b), or under conditions that readily generate mast cells from total bone marrow17 (data not shown). By contrast, inclusion of IL-33 and IL-7 into the cultures induced substantial expansion of nuocytes (Fig. 3b). Addition of IL-25 to IL-33 + IL-7 culture conditions did not change the growth rate of nuocytes (Fig. 3b).

The cultured nuocytes maintained the expression of the majority of cell surface markers, including high levels of CD45 (data not shown) and ICOS, T1/ST2, IL-17BR and IL-7Rα (Supplementary Fig. 8), and did not differentiate into any of the currently known leukocyte lineages even after 15 days in culture (Fig. 1e and data not shown). All nuocytes expressed IL-13, with more than 70% also secreting IL-5, though less than 5% produced IL-4 (Fig. 3c). Analysis of nuocyte culture supernatants revealed the additional substantial secretion of IL-6, IL-10, and GM-CSF (Supplementary Fig. 9).

Strikingly, adoptive transfer of nuocytes into *Il17br*$^{-/-}$ mice re-established many of the features of IL-25-evoked type-2 immune responses (Fig. 3d and e) that are normally absent in these mice (Supplementary Fig. 3e-g). Crucially, the adoptive transfer of nuocytes did not induce any spontaneous inflammation in *Il17br*$^{-/-}$ recipients but restored their ability to respond to subsequent IL-25 administration (Fig. 3d and e). Cellular infiltrate of the peritoneal lavage, characterised by eosinophilia (Fig. 3d), and intestinal goblet cell hypertrophy and hyperplasia, were restored in *Il17br*$^{-/-}$ mice that received nuocytes, as compared to controls (Fig. 3e).

To investigate whether nuocytes play a critical role in co-ordinating immunity to helminth infection, we sought to restore protective immunity to *Il17br*$^{-/-}$ mice in response to *N. brasiliensis* through the adoptive transfer of wildtype (IL-25 responsive) nuocytes. Four days p.i., all infected animals had equivalent intestinal worm burdens (Fig. 4a), demonstrating that the transfer of nuocytes did not prevent establishment of infection. Strikingly, the majority of the infected *Il17br*$^{-/-}$ animals that received nuocytes had completely expelled their worms by day 11 post-infection, similar to wildtype controls, (Fig. 4a). This contrasted with the *Il17br*$^{-/-}$ mice that had not received nuocytes, and had burdens of greater than 50 worms at the same time point (Fig. 4a). The transfer of nuocytes also restored the numbers of nuocytes in the tissues at day 11 p.i. (Fig. 4b), and restored the early eosinophil response at day 6 p.i., but did not appreciably alter the levels of basophils at this time point (Supplementary Fig. 7a and b). Furthermore, adoptive transfer of wildtype nuocytes into the combined *Il17br*$^{-/-}$*Il1rl1*$^{-/-}$ mice also resulted in the restoration of *N. brasiliensis* expulsion (Fig. 4c).

We have shown previously that IL-13, amongst the type-2 cytokines, is essential for the rapid eradication of *N. brasiliensis*13,18. We now demonstrate by transferring wildtype nuocytes into *Il4*$^{-/-}$*Il13*$^{-/-}$ mice that, even when nuocytes are the only IL-13-secreting cells, they are capable of inducing worm expulsion (Fig. 4d). To further test the importance of nuocyte-derived IL-13 on the kinetics of *N. brasiliensis* expulsion, nuocytes were prepared from *Il13*$^{-/-}$ animals and transferred into *N. brasiliensis*-infected *Il17br*$^{-/-}$ animals. In contrast to the wildtype nuocytes, IL-13-deficient nuocytes failed to mediate worm expulsion, with mice continuing to harbour high numbers of intestinal worms at day 11 p.i. (Fig. 4e).

In vitro antigen re-stimulation of mLN cells from *N. brasiliensis*-infected *Il17br*$^{-/-}$ mice revealed delayed T cell-derived IL-13 secretion that was absent at day 4 p.i. However, the continuing presence of intestinal worms and the resulting antigen burden led to a robust cytokine response by day 11 p.i. (Fig. 4f), as reported previously[19]. We also observed fewer IL-13eGFP$^+$ T cells in both *Il1rl1*$^{-/-}$ mice and *Il17br*$^{-/-}$ mice at five days p.i (data not shown). However, following adoptive transfer of wildtype or *Il13*$^{-/-}$ nuocytes into *Il17br*$^{-/-}$ mice, antigen-specific T cell production of IL-13 was restored (Fig. 4f). As expected, since *Il13*$^{-/-}$ nuocytes failed to induce worm expulsion, the duration of antigen-specific T cell responses was prolonged (Fig. 4f). Thus, nuocytes are capable of enhancing T cell cytokine production, and nuocytes augment T cell responses independently of IL-13.

Expulsion of *N. brasiliensis* is a T cell-dependent process, but neither T cell-derived IL-4 or IL-13 are necessary for worm expulsion[15], indicating that an alternative cell source is responsible for the production of the IL-13 necessary for worm expulsion. It was not surprising then that nuocytes were unable to induce worm expulsion in *Rag2*$^{-/-}$ mice (Fig 4g). However, analysis of nuocyte numbers in the mLN of *N. brasiliensis*-infected *Rag2*$^{-/-}$ mice revealed that, despite rapid early nuocyte expansion by day 4 p.i. (Fig. 4h), nuocyte numbers were not maintained in the absence of T cells, falling to uninfected levels as assessed on days 6 and 11 p.i. (Fig. 4h), despite the continued presence of intestinal worms (Fig. 4g). This suggests that T cells (or possibly B cells, though B cells have been shown to be dispensable for worm expulsion[15]) mediate prolonged nuocyte expansion, migration or survival through an as yet unknown mechanism that requires further investigation. Our data demonstrate that a dialogue exists between T cells and nuocytes, and that this is necessary for robust *N. brasiliensis* expulsion.

Thus, nuocytes clearly provide a critical effector mechanism, through their provision of the IL-13, required to induce helminth expulsion. The ability of nuocytes to expand rapidly in response to two potent initiators of type-2 immunity (IL-25 and IL-33), and in response to helminth infection, their presence at multiple immuno-surveillance sites around the body, their capacity to secrete high levels of IL-13 and IL-5, and their ability to enhance T cell responses, shows them to be highly specialised type-2 regulatory cells. The characterisation of nuocytes will now allow assessment of their roles in other immune responses and disease pathologies including allergic asthma.

METHODS SUMMARY

Adoptive Transfers

Purified nuocytes were cultured for 2 - 5 days in RPMI supplemented with 10 ng/ml IL-33 and 10 ng/ml IL-7. Cells were washed and injected intravenously in PBS via the tail vein at 0.5×10^6 cells/mouse. Adoptive transfer was performed four hours after infection with *N. brasiliensis*, or 12 hours before the first cytokine administration of IL-25 i.p.

ONLINE METHODS

Animals

BALB/c mice were purchased from Charles River Laboratories (Margate, Kent, UK) as required. *Rag2*$^{-/-}$ mice[20], on a BALB/c background, were provided by Jean Langhorne (NIMR, London). *Il13*$^{-/-}$ mice[13] and *Il4*$^{-/-}$*Il13*$^{-/-}$ mice[18] were on a BALB/c background. *Ja18*$^{-/-}$ mice[21], on a C57BL/6 background. *Kit*$^{Wsh/Wsh}$ mice[22], on a C57BL/6 background, were provided by Catherine Lawrence (University of Strathclyde). In individual experiments all mice were matched for age, gender and background strain. Mice were maintained in specific pathogen-free conditions. All animal experiments undertaken in this study were done so with the approval of the UK Home Office.

Il13eGFP mice

The *Il13eGFP* mice were generated by recombineering[23],[24] (Supplementary Information). Neomycin negative, Cre-recombinase negative mice were backcrossed onto the BALB/c and C57BL/6 backgrounds. Genotyping of Il13eGFP mice used PCR primers ASEQ3755 (Forward, 5′-tcaacaggctaaggccacaagcc-3′), ASEQ4030 (Forward, 5′-CATGGTCCTGCTGGAGTTCGTG-3′) and ASEQ3958 (Reverse, 5′-GCTTCGTCTGTCACTCACACAGG-3′), giving a wildtype product of 300 bp and a targeted product of 522 bp.

Il17br$^{-/-}$ mice

The replacement vector was designed to replace exons 2 and 3 of the *il17br* gene (a region encoding 56 amino acids of IL-17BR) with a neomycin resistance gene (Supplementary Information). Targeted BALB/c embryonic stem (ES) cell clones were used to generate the line on a BALB/c background. Genotyping was performed by PCR using primers (5′-TTGCTGATCTTGGCTGCATCGTGC-3′), (5′-AGCAGGGCTTGCATCTGAATGCCT-3′) and (5′-CTATCAGGACATAGCGTTGGCTACC-3′) that give a product of 600 bp for the wild-type allele and 400 bp for the targeted allele.

Generation of monoclonal anti-IL-17BR antibody (Clone D9.2)

Il17br$^{-/-}$ mice were immunised intraperitoneally with murine IL-17Br/Fc fusion protein (R&D Systems) and monoclonal anti-IL-17BR antibodies generated by standard protocols (Supplementary Information).

IL-25 and IL-33 administration

0.4 μg per dose of recombinant mouse IL-25 or recombinant mouse IL-33 (R&D Systems) in PBS was administered daily for 3 days intraperitoneally. Mice were sacrificed 24 hours later and tissues harvested for analysis. Control animals received PBS only.

Fluorescence-activated cell analysis

Mouse tissue cell suspensions at 2×10^8 cells/ml were incubated with purified anti-Fc receptor blocking antibody (anti-CD16/CD32) before addition of the specific antibodies. Cell surface markers were stained using a combination of FITC-, PE-, PE-Cy7-, APC-conjugated and biotin-conjugated monoclonal antibodies (see Supplementary Information). In each experiment the appropriate isotype control monoclonal antibodies and single conjugate controls were also included. Samples were analysed using a Becton Dickinson FACScalibur flow cytometer running CellQuest acquisition and analysed using FlowJo software (version 8.8.3, Tree Star Inc. OR, USA).

Fluorescence-activated cell sorting of nuocytes

Spleen cells prepared from IL-25-treated mice were depleted of lineage$^+$ cells prior to cell sorting by incubation with biotin-conjugated anti-CD3, anti-CD19, anti-CD11b and anti-FcεRI antibodies before removal of antibody-bound cells by magnetic separation using Dynabeads (Invitrogen). Lineage-depleted cells were stained with PE-conjugated antibodies against CD4, CD8, B220, TER-119 and CD11b, a FITC-conjugated antibody against CD45 and an APC-conjugated antibody against ICOS. PE$^-$, FITC$^+$, APC$^+$ cells were collected using a Mo-flo cell sorter, and purity checked by staining with lineage antibodies and antibodies against IL-17BR and T1/ST2.

Nuocyte cytokine/chemokine profile analysis

Supernatants collected from day 7 cultured nuocytes ($\sim 2 \times 10^6$ cells/ml) were analyzed by bioplex assay (Milliplex MAP mouse cytokine/chemokine 22-plex, Millipore). Supernatants were assayed according to manufacturer's instructions. Data were collected with the Bio-Plex 200 system, analyzed in Excel and graphed with GraphPad Prism 4.0 software. Statistical significance was determined by one-way ANOVA with Tukey Post Test. $p < 0.05$ was considered significant.

Helminth infection and antigen restimulation

Mice were inoculated subcutaneously with 300 viable third-stage *N. brasiliensis* larvae. mLN cells were stimulated *in vitro* at 2×10^6 cells/ml with 50 μg/ml of parasite antigens (*N. brasiliensis* excretory/secretory antigen) for 72 hours. Supernatants were collected and analyzed for IL-13 by Quantikine ELISA (R&D Systems).

Immunofluorescence on cryosections and confocal microscopy

Cryosections were prepared (Supplementary Information) and incubated with conjugated antibodies: anti-mouse CD11b-Pacific Blue (clone M1/70, eBioscience), anti-mouse B220-Pacific Blue (clone RA3-6B2, eBioscience), anti-mouse CD4-biotin (clone RM4-5, Biolegend), anti-mouse CD3-Pacific Blue (clone eBio500A2, eBioscience), anti-mouse SIGN-R1-AlexaFluor 647 (clone eBio22D1, eBioscience), anti-GFP (rabbit IgG, Invitrogen). Sections were then rinsed and incubated with streptavidin conjugated with AlexaFluor 546 (Invitrogen) and/or anti-rabbit-AlexaFluor 488 (goat IgG, Invitrogen). 7-amino-actinomycin (7-AAD, eBioscience) was included in the last incubation to stain nuclei. Finally, samples were mounted with Vectashield (Vector Labs). Images were taken on a Carl-Zeiss inverted microscope (LSM 710) and processed with ZEN 2008 (Carl-Zeiss).

Microarray data analysis

Mouse Genome 430 2.0 GeneChips were used to analyse the gene expression of freshly isolated or 9 days in vitro expanded nuocytes from wildtype mice. Raw expression data were initially imported into R and Bioconductor software for quality assessment using the affyQCReport package. Nuocyte data were combined with publically available immune cells datasets with the same GeneChips. These include mouse B cells (n=3), CD11b+ dendritic cells (DCs) (n=2), CD8+ DCs (n=2), pDC (n=2), CD8+ cells (n=2), NK cells (n=2) (all from Gene Expression Omnibus (GEO), (http://www.ncbi.nlm.nih.gov/projects/geo), under accession ID GSE9810), CD4+ cells (n=2, GEO accession GSM44979 and GSM44982), mast cells (n=2, GEO accessions GSM258711 and 258712) and granulocytes (n=2, GEO accessions GSM149595 and GSM149596). Datasets for macrophages (n=3) were obtained from the National Cancer Institute caArray (http://caarray.nci.hih.gov/). All data were imported into R and Bioconductor software using affy package. Background correction, normalization, PM correction and summarisation of the data were performed with the methods: rma, quantiles, pmonly and medianpolish respectively, using the affy function expresso. The statistical properties of all the arrays after the pre-processing step were examined and confirmed to be very similar. Cluster analysis was then performed using the clustering algorithm divisive analysis clustering (Diana) which is a function in the cluster package from bioconductor.

Statistical Analysis

Graph Pad Prism was used to calculate the Standard Deviation (SD) between experimental samples when each experimental group contained an equal number of data sets. In the case where different numbers of data sets existed in each experimental group the Standard Error of the Mean (SEM) was used. When data were normally distributed and when two

independent variables were being analysed, 2-way ANOVA with Bonferroni post-analysis was performed. In all other instances statistical differences between groups were calculated using Student's t test where a p value of < 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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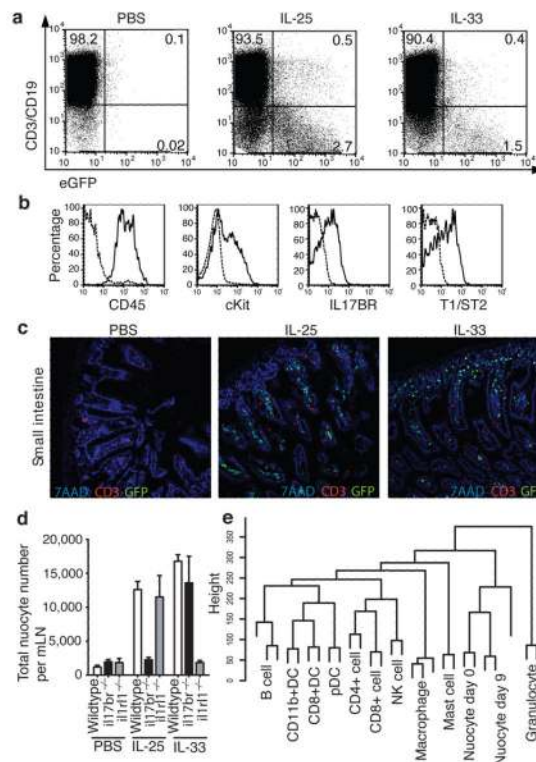
Figure 1. IL-25 and IL-33 induce IL-13-producing nuocytes
a, Detection of *Il13eGFP*^{+} NBNT cells in mLN of IL-25 or IL-33-treated mice. **b,** Cell
surface marker expression of *Il13eGFP*^{+} NBNT cells in mLN following IL-25
administration. **c,** Immunofluorescence detection of *Il13eGFP*^{+} cells in small intestine of
IL-25 and IL-33 treated mice. **a** – **c,** Data representative of 5 experiments with >3 mice per
group. **d,** Nuocyte number. Data representative of two independent experiments with >4
mice per group. **e,** Cluster analysis for freshly isolated nuocytes (*ex vivo*) or day 9 *in vitro*
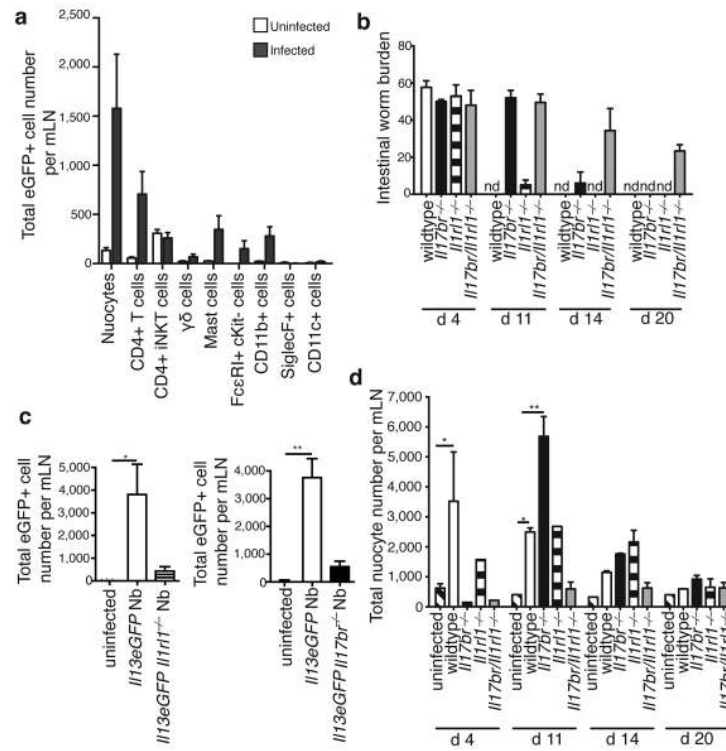expanded nuocytes (single data sets are shown for clarity).

Figure 2. IL-25 and IL-33 play partially redundant roles for nuocyte induction and worm expulsion

a, Quantification of *Il13eGFP*+ cells five days p.i. with *N. brasiliensis*. Data are representative of two independent experiments with >5 mice per group. **b,** Intestinal worm burden of *N. brasiliensis*-infected mice. c, Quantification of *Il13eGFP*+ cells in *N. brasiliensis*-infected mice at day 5 p.i. Data are representative of two independent experiments with >5 mice per group. d, Quantification of nuocytes in *N. brasiliensis*-infected mice. * = $p < 0.05$, ** = $p < 0.01$. Data are representative of two independent experiments with >5 mice per group.
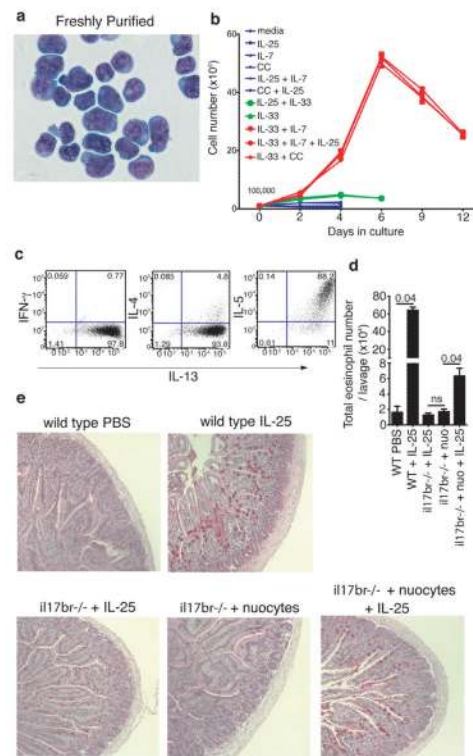
Figure 3. Adoptive transfer of cultured nuocytes into *il17br*^{−/-} mice restores an IL-25-responsive phenotype

a, Morphology of Giemsa-stained nuocytes. **b,** Quantification of nuocyte growth *in vitro*. CC, cytokine cocktail. **c**, Flow cytometric analysis of interferon (IFN)-γ, IL-4, IL-5 and IL-13 intracellular staining of nuocytes after 7 days culture with IL-7 and IL-33. **a – c,** Data are representative of three independent experiments. **d**, Quantification of eosinophil infiltration of the peritoneal cavity following nuocyte (nuo) transfer. **e**, Transverse histological jejunum sections stained with Periodic acid-Schiff (PAS) for goblet cells. Data are representative of two independent experiments with >5 mice per group.
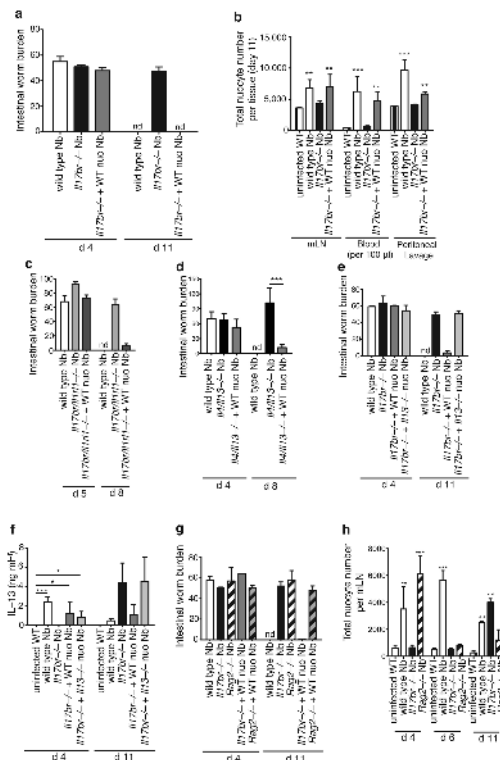
Figure 4. Adoptive transfer of wildtype nuocytes, but not IL-13-deficient nuocytes, restores rapid worm expulsion in *N.brasiliensis* infected *il17br*$^{-/-}$ mice

a, Intestinal worm burdens. **b,** Quantification of nuocyte numbers in tissues. Data are representative of three independent experiments with >6 mice per group. **c - d**, Intestinal worm burdens. Data are from single experiments with 6 - 7 mice per group. **e,** Intestinal worm burdens. **f,** *N. brasiliensis* antigen-specific IL-13 production. **g,** Intestinal worm burden. **h,** Quantification of nuocyte numbers. **e – h**, Data are representative of two independent experiments with >6 mice per group. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$.