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Autocrine IL-17A–IL-17RC neutrophil activation in fungal infections is regulated by IL-6, IL-23, RORγt and Dectin-2

Patricia R. Taylor[1], **Sanhita Roy**[1,*], **Sixto M. Leal Jr.**[1,*], **Yan Sun**[1], **Scott J. Howell**[1], **Brian A. Cobb**[2], **Xiaoxia Li**[3], and **Eric Pearlman**[1,#]

[1]Department of Ophthalmology and Visual Sciences, Case Western Reserve University

[2]Department of Pathology, Case Western Reserve University

[3]Department of Immunology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH

Abstract

Here we identified a population of bone marrow neutrophils that constitutively express RORγt and which can produce and respond to IL-17A (IL-17). IL-6, IL-23 and RORγt, but not T cells or NK cells, are required for IL-17 production in neutrophils. IL-6 and IL-23 induced IL-17RC and Dectin-2 expression in neutrophils, and expression of IL-17RC was augmented by *Aspergillus* and Dectin-2 activation. Autocrine IL-17A–IL-17 receptor activity induced production of reactive oxygen species (ROS), and increased fungal killing *in vitro* and in a model of *Aspergillus* keratitis. Human neutrophils also expressed RORγt, and induced IL-17A, IL-17RC and Dectin-2 expression following IL-6 and IL-23 stimulation. These findings identify a population of human and murine neutrophils that exhibit autocrine IL-17 activity, and which likely contribute to the etiology of microbial and inflammatory diseases.

Interleukin 17 (IL-17A, here IL-17) mediates the severity of autoimmune and inflammatory disease and contributes to protection against bacterial and fungal infections [1, 2]. Individuals with impaired IL-17 responses due to production of anti-IL-17 auto-antibodies, mutations in STAT3 or mutations in STAT1 that affect IL-23 production exhibit increased susceptibility to mucocutaneous candidiasis [3-7]. Although T_H17 cells are considered to be the major source of IL-17, NKT cells, γδ T cells and innate lymphoid cells produce IL-17 more rapidly than T cells due to constitutive expression of the RORγt transcription factor [8]. Neutrophils have also been identified as a source of IL-17 in human psoriatic lesions [9] and in several murine models of infectious and autoimmune inflammation [10-13]. Elevated IL-17 expression

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[#]Corresponding author: Eric Pearlman, 10900 Euclid Ave, Cleveland OH 44106, Tel: 216 368 1856, Fax: 216 368 3171, Eric.Pearlman@case.edu.

[*]These authors contributed equally to this work

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was also observed in patients with corneal ulcers caused by filamentous fungi, where neutrophils were the predominant infiltrating cells [14].

In the current study, we present data showing that human peripheral blood neutrophils and murine bone marrow neutrophils express IL-17A transcripts and protein following stimulation with IL-6 and IL-23. We used RORγt reporter mice (*Rorc*$^{+/GFP}$) [15] to identify a population of neutrophils that constitutively express this transcription factor, which has until now been associated only with cells derived from the lymphoid lineage [8, 15, 16], and show that RORγt deficient *Rorc*$^{GFP/GFP}$ neutrophils did not produce IL-17. We also demonstrate that IL-6 and IL-23 induced the expression of IL-17RC on human and murine neutrophils, which was further increased in the presence of *Aspergillus* hyphae by a Dectin-2 – dependent pathway. Finally, activation of the IL-17RA/IL-17RC receptor by endogenous or exogenous IL-17 activation enhanced the production of reactive oxygen species (ROS), which mediated increased fungal killing *in vitro* and in a murine model of *Aspergillus* corneal infection. The role of IL-17 in infection and inflammation is currently thought to involve activation of IL-17RA and IL-17RC expressing fibroblasts and epithelial cells to produce CXC chemokines and pro-inflammatory cytokines that mediate recruitment of neutrophils and release of cytotoxic mediators such as reactive oxygen species (ROS). In the current study, we identified a population of neutrophils that produce and utilize IL-17 in an autocrine manner to enhance ROS production and anti-fungal activity.

Results

IL-17 production by neutrophils is dependent on IL-6 and IL-23

To determine if bone marrow neutrophils can be induced to express IL-17 *in vivo*, we injected C57BL/6, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ and *Il6*$^{-/-}$mice subcutaneously with swollen, heat-killed *Aspergillus fumigatus* conidia. Three days later, IL-17 production in total bone marrow cells from naïve and from these ‘primed’ mice were examined by flow cytometry. 27.8% of total bone marrow cells in naïve C57BL/6 mice were Ly6G^{+} neutrophils as indicated by reactivity with NIMP-R14 antibody, and there were no cells exhibiting intracellular IL-17 (Fig. 1a). In contrast, 6.3% of cells in *Aspergillus* – primed mice were IL-17^{+}, all of which were also NIMP-R14^{+}. NIMP-R14^{+} bone marrow cells from primed *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice, which do not have T cells or natural killer (NK) cells, were also IL-17^{+} after priming, indicating that T cells and NK cells are not required for IL-17 production by neutrophils. CD3^{+} or NK1.1^{+} cells isolated from the spleens of C57BL/6 mice 3 days after *Aspergillus* infection did not express IL-17, although CD3^{+}IL-17^{+} cells were detected in immunized mice 10 days after subcutaneous injection (Supplementary Fig. 1a). In contrast, IL-17^{+} bone marrow cells were not detected in IL-6$^{-/-}$ mice, indicating an essential role for this cytokine in neutrophil IL-17 production. To assess IL-17 gene expression in bone marrow neutrophils, we also examined *Il17-GFP* reporter mice, which express functional IL-17. We found that 6.7% of total bone marrow cells in primed, but not naïve reporter mice, were GFP^{+} NIMP-R14^{+} (Fig. 1a).

Neutrophils isolated from the bone marrow of naïve C57BL/6 mice by gradient centrifugation were >99% NIMP-R14^{+}, and had a characteristic polymorphonuclear morphology (Fig. 1b). This population was also 1A8^{+} CD11b^{+}, and was negative for F4/80

and lymphoid cell markers (Supplementary Fig. 1b). Further, neutrophils isolated from primed, but not naïve *Il17*-GFP mice had intracellular IL-17 (Fig. 1c). Individual GFP expressing cells were also detected by confocal microscopy (Fig. 1d) and by Imagestream analysis (Supplementary Fig. 1c). As a second approach to assess IL-17 gene expression, NIMP-R14$^+$ bone marrow cells from C57BL/6 mice were sorted by flow cytometry and gene expression was examined by quantitative PCR. Cell sorted neutrophils from *Aspergillus*-primed, but not naïve mice expressed IL-17A mRNA and protein (Supplementary Fig. 1d,e).

Because IL-6, IL-23, IL-1β and TGF-β mediate IL-17 expression in lymphoid cells, we examined their role in IL-17 production by neutrophils. Serum from primed C57BL/6 and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice showed elevated amounts of IL-6 and IL-23 compared to serum from naïve mice (Fig. 1e). Similarly, IL-6 and IL-23 (and IL-1β and TGF-β) were produced by splenocytes from naïve C57BL/6 and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice following 18h incubation with *Aspergillus* hyphal extract (AspHE) (Fig. 1f), indicating that neither T cells nor NK cells are required for production of these cytokines.

To ascertain if there is a role for these cytokines in neutrophil IL-17 expression, isolated neutrophils were incubated for 1 h with splenocyte supernatants in the presence of neutralizing antibodies, and IL-17 expression was examined by quantitative PCR. IL-17 PCR products were detected after stimulation with splenocyte supernatants from primed, but not naïve C57BL/6 mice; further, neutralization of either IL-6 or IL-23 ablated IL-17 expression, whereas antibodies to IL-1β or TGF- β had no effect (Fig. 1g). (ct scores are shown in (Supplementary Table 1) . Taken together, these results demonstrate that expression of IL-17 by neutrophils is dependent on IL-6 and IL-23, but not IL-1β or TGF-β, and that neither T cells nor NK cells are required to induce IL-17 production in neutrophils.

RORγt mediates neutrophil IL-17 expression

Because the RORγt transcription factor mediates IL-17 production by T_H17, γδ T cells and innate lymphoid cells [8, 17, 18], we examined its role in neutrophil IL-17 production using homozygous *Rorc*$^{GFP/GFP}$ mice that express eGFP under the control of the *Rorc* promoter, and which do not produce functional RORγt [15]. We also used heterozygous *Rorc*$^{+/GFP}$ littermate controls, which express functional RORγt [15], to detect *Rorc* expression and RORγt protein in neutrophils. Approximately 7% of total GFP-expressing bone marrow cells from naïve *Rorc*$^{+/GFP}$ and *Rorc*$^{GFP/GFP}$ mice were NIMP-R14$^+$ (Fig. 2a upper panels), indicating constitutive expression of this gene in neutrophils. NIMP-R14$^+$ GFP$^+$ cells were also detected by Imagestream, and *Rorc* mRNA was expressed in C57BL/6 neutrophils (Supplementary Fig. 2a,b). Intracellular staining using RORγt antibodies showed 5.6% of C57BL/6 neutrophils and 6.4% of *Rorc*$^{+/GFP}$ neutrophils were RORγt$^+$ (Fig. 2a, lower panels), thereby demonstrating that RORγt is constitutively expressed in a sub-population of neutrophils. *Rorc*$^{GFP/GFP}$ neutrophils did not express RORγt (Fig. 2a).

To characterize this neutrophil population further, we examined expression of IL-6 and IL-23 receptors on bone marrow neutrophils isolated from naïve C57BL/6 mice by density centrifugation. Although 26.7% bone marrow neutrophils expressed IL-6R, only 18% expressed the gp130 subunit, and 17.8% and 19.3% expressed the IL-23R and IL-12Rβ1

subunits (Fig. 2b). IL-23R gene expression was also detected by quantitative PCR (Supplementary Fig. 2c,d).

To determine the role of RORγt in IL-17 production in neutrophils, bone marrow neutrophils from C57BL/6, *Rorc*$^{+/GFP}$ and *Rorc*$^{GFP/GFP}$ mice were incubated for 3h with 20μg/ml murine (rm)IL-6 and 2 μg/ml rmIL-23 (IL-6+IL-23) (Stimulation with IL-6 or IL-23 alone did not induce IL-17 mRNA expression, Supplementary Fig. S2e-g). Intracellular IL-17 was not detected in neutrophils from naïve mice; however, IL-6+IL-23 stimulated C57BL/6 and *Rorc*$^{+/GFP}$ neutrophils expressed IL-17, whereas IL-17 was not detected in *Rorc*$^{GFP/GFP}$ neutrophils (Fig. 2c, d). IL-6+IL-23 also induced IL-17 gene expression in C57BL/6 and *Rorc*$^{+/GFP}$, but not in *Rorc*$^{GFP/GFP}$ neutrophils (Fig. 2e, Supplementary Table 1). Together, these observations identify a population of bone marrow neutrophils that constitutively express RORγt and IL-6 and IL-23 receptors, and which produce IL-17 following stimulation with IL-6 and IL-23. These data also show that IL-6+IL-23 - induced IL-17 expression by neutrophils is completely dependent on RORγt.

IL-6 and IL-23 induce RORγt nuclear translocation

To examine further the role of RORγt in neutrophils, bone marrow neutrophils from naïve C57BL/6 mice were incubated with splenocyte supernatants containing IL-6 and IL-23, or with recombinant IL-6 or IL-23 separately, and RORγt protein was examined in total cell lysates and in nuclear extracts by immunoblot analysis. RORγt was present in total cell lysates from unstimulated neutrophils, but was only detected in nuclear extracts after incubation with splenocyte supernatants containing IL-6 and IL-23 (Fig. 3a, b). RORγt was also detected in nuclear extracts after incubation with rIL-6 or rIL-23; however, expression was higher after stimulation with IL-6+IL-23 (Fig. 3c). After 1h incubation with IL-6+IL-23, RORγt was also detected in the nucleus by confocal microscopy (Fig. 3d).

To assess the RORγt transcriptional activity in IL-6+IL-23-stimulated neutrophils, nuclear extracts were examined by EMSA using a biotinylated oligonucleotide probe corresponding to a putative RORγt binding region on the IL-17 promoter [19] (sequence in Methods). A biotinylated product was detected in IL-6+IL-23 – stimulated neutrophils, which showed a mobility shift following incubation with anti-RORγt (Fig. 3e). Further, binding of the biotinylated probe was inhibited in the presence of a competitive, unlabeled (cold) probe, and was not detected after incubation with a labeled (mutant) probe that had three base substitutions. Together, these findings demonstrate that IL-6+23 induces RORγt translocation to the nucleus, and is consistent with RORγt binding to the IL-17 promoter.

Human neutrophils express RORγt and produce IL-17

To ascertain if IL-6+IL-23 activates RORγt and IL-17 production in human neutrophils, a highly purified population of peripheral blood neutrophils was isolated by gradient centrifugation (Supplementary Fig. 3a), and incubated with supernatants from AspHE – stimulated peripheral blood mononuclear cells (PBMC). IL-6, IL-23, IL-1β and TGF-β were detected in supernatants from AspHE-stimulated PBMC (Fig. 4a). Further, IL-17 gene expression was elevated in neutrophils stimulated 1h with supernatants from AspHE-

stimulated PBMCs, although expression was inhibited in the presence of anti–IL-6 or anti–IL-23 (Fig. 4b, Supplementary Table 1).

Consistent with these findings, IL-6+IL-23 – stimulated human neutrophils exhibited increased IL-17 gene expression (Fig. 4c), IL-17 protein in total cell lysates (Fig. 4d), and intracellular IL-17 in ∼80% of the population (Fig. 4e). Stimulation with IL-6 or IL-23 alone was not sufficient to induce IL-17 mRNA or protein expression in human neutrophils (Supplementary Fig. 3b,c). Intracellular RORγt, and cell surface IL-6R and IL-23R were expressed in 75-77% of unstimulated peripheral blood neutrophils, and were co-expressed with intracellular IL-17 following IL-6+IL-23 stimulation (Fig. 4f, g and Supplementary Fig. 3d-g). Together, these findings demonstrate that, similar to murine neutrophils, RORγt and IL-6 and IL-23 receptors are expressed in a subset of human peripheral blood neutrophils, and that IL-6 and IL-23 are necessary and sufficient for IL-17 gene expression in these cells.

IL-17RC expression is induced by IL-6+23 and by Dectin-2

The IL-17A and IL-17F receptors comprise IL-17RA and IL-17RC subunits, which signal through Act1 [20, 21]. Although unstimulated human peripheral blood neutrophils express IL-17RA and Act1[22, 23], neither human nor murine neutrophils express IL-17RC [22, 23]. Consistent with these reports, we found that bone marrow neutrophils from naïve C57BL/6 and IL-17A-/- mice expressed IL-17RA, but not IL-17RC (Fig. 5a). However, IL-17RC gene expression was elevated after 1h incubation with IL-6+IL-23, and was further increased in the presence of AspHE but not rIL-17 (Fig. 5a, b). Similarly, IL-17RC gene and cell surface expression were detected in human neutrophils incubated with IL-6+IL-23, and were further elevated with AspHE (Fig. 5c,d). IL-17RC was not detected following incubation with either IL-6 or IL-23 alone (Supplementary Fig. 4a).

Because AspHE enhanced IL-17RC expression, we assessed if this was mediated by Dectin-1 and Dectin-2, which recognize β-glucan and α-mannan, respectively, in the fungal cell wall [24, 25]. Naïve C57BL/6 neutrophils expressed Dectin-1 (*Clec7a)*, which was increased with IL-6+IL-23 (Fig. 5e). In contrast, Dectin-2 (*Clec4n)* was not expressed in unstimulated neutrophils, but was induced at the RNA and cell surface levels by IL-6+IL-23 (Fig. 5e). Similarly, peripheral blood human neutrophils only expressed Dectin-2 (*Clec6a*) after incubation with IL-6+IL-23 (Fig. 5f and Supplementary Fig. 4b).

Bone marrow neutrophils from C57BL/6, *Clec7a* $^{-/-}$ and *Clec4n* $^{-/-}$ mice were then stimulated with IL-6+IL-23 and AspHE, and IL-17RC gene and cell surface expression were assessed. We found that AspHE – induced IL-17RC expression was also elevated in *Clec7a* $^{-/-}$ neutrophils, whereas IL-17RC expression in AspHE – stimulated *Clec4n* $^{-/-}$ neutrophils was not increased above that induced with only IL-6+IL-23 (Fig. 5g). These findings demonstrate that Dectin-2 mediates the increased IL-17RC expression in response to fungal hyphae.

IL-6+IL-23 – stimulated *Rorc*$^{GFP/GFP}$ neutrophils expressed IL-17RC and Dectin-2 to the same extent as littermate *Rorc*$^{+/GFP}$ neutrophils (Fig. 5h,i), indicating that expression of these receptors is not dependent on RORγt. *Rorc*$^{GFP/GFP}$ neutrophils also express IL-23R

(Supplementary Fig. 4c). Finally, we found no difference in cell surface IL-17 expression by IL-6+IL-23 – stimulated *Clec7a* $^{-/-}$ and *Clec4n* $^{-/-}$ compared with C57BL/6 neutrophils (Fig. 5j), demonstrating that IL-17 expression is not regulated by Dectin-1 or Dectin-2.

Autocrine IL-17 – IL-17R – mediated fungal growth inhibition

As neutrophil NADPH oxidase function and reactive oxygen species (ROS) production limit the growth of *Aspergillus* hyphae [26], we examined the role of IL-17A and IL-17 receptor in ROS production and fungal killing. Bone marrow neutrophils from *Il17a*$^{-/-}$ and *Il17rc*$^{-/-}$ mice were stimulated with IL-6+IL-23, and incubated with *A. fumigatus* hyphae. We also examined neutrophils from *Cybb*$^{-/-}$ (chronic granulomatous disease) mice, which do not express the GP91 subunit of NADPH oxidase [27]. ROS production was measured by intracellular CFDA, and fungal growth was quantified by fluorimetry of RFP – expressing *Aspergillus* hyphae as described [26, 28]. To ensure that the mutations did not affect expression of other relevant genes, we showed that IL-6+IL-23 – stimulated *Il17a*$^{-/-}$ neutrophils expressed normal levels of IL-17RC, *Il17rc*$^{-/-}$ neutrophils expressed normal levels of IL-17, and *Cybb*$^{-/-}$ neutrophils expressed normal levels of IL-17 and IL-17RC (Fig. 6a,b). As previously reported [26], neutrophils from naïve C57BL/6 mice produced ROS after incubation with *Aspergillus* hyphae (Fig. 6c, upper panel); however, ROS production was elevated in C57BL/6 neutrophils from primed mice, and from naïve mice stimulated *in vitro* with IL-6+IL-23, and was not further increased following incubation with rIL-17A (Fig. 6c). In contrast, ROS production by *Il17a*$^{-/-}$ neutrophils was only increased after further stimulation with rIL-17, and was blocked in the presence of antibodies to IL-17RC (Fig. 6c). ROS production was not increased in *Il17rc*$^{-/-}$ neutrophils, even in the presence of rIL-17A (Fig. 6c), demonstrating that IL-17RC expression is essential for IL-6+IL-23 – induced ROS production. ROS was not detected in IL-6+IL-23 – stimulated *Cybb*$^{-/-}$ neutrophils (Fig. 6c and Supplementary Fig. 5).

Conversely, hyphal growth was impaired following incubation with IL-6+IL-23 – stimulated C57BL/6 neutrophils and *Il17a*$^{-/-}$ neutrophils + rIL-17 (Fig. 6c, lower panel), whereas RFP *Aspergillus* incubated with IL-6+IL-23 – *Il17rc*$^{-/-}$ neutrophils was the same as with unstimulated cells, and the same as medium control when incubated with *Cybb*$^{-/-}$ neutrophils (Fig. 6c).

IL-6+IL-23 – stimulated human peripheral blood neutrophils exhibited the same phenotype as murine neutrophils: ROS production and inhibition of hyphal growth was dependent were elevated compared with unstimulated neutrophils, and was reversed by IL-17RC blockade, and ablated in the presence of the NADPH oxidase inhibitor DPI (Fig. 6d). Overall, these data indicate that IL-6+IL-23 – induced ROS production and fungal killing by stimulated neutrophils is dependent on IL-17 and a functional IL-17 receptor, and is mediated by NADPH oxidase.

IL-17 and IL-17R – dependent fungal growth inhibition *in vivo*

To determine if IL-17 – producing neutrophils regulate fungal growth *in vivo*, C57BL/6 and *Il17-GFP* mice were primed by subcutaneous injection of heat – killed swollen conidia to generate IL-17 producing neutrophils. After 72h, live RFP-expressing *Aspergillus* conidia

were injected into the corneal stroma and 24h later, RFP expressing hyphae in the cornea were quantified by image analysis as described [26]. Primed, infected C57BL/6 and *Il17-GFP* mice had significantly less RFP hyphae than infected mice that had not been primed (Fig. 7a,b). Also, although there were no differences in total neutrophils in infected corneas (Fig. 7c), IL-17 expressing neutrophils were only detected in corneas of primed, infected mice (Fig. 7a,d, Supplementary Fig. 6a). IL-17-producing and non-producing neutrophils from infected corneas were 95% viable (Supplementary Fig. 6b).

As a second approach to examine the role of IL-17 producing neutrophils on pathogenic fungi, we used an adoptive transfer model in which donor neutrophils are injected intravenously into recipient *Cd18*$^{-/-}$ mice [26]. CD18 deficient neutrophils cannot bind to ICAM-1 on capillary endothelial cells, and therefore cannot migrate into infected or inflamed tissues [29]. Bone marrow neutrophils from naïve C57BL/6 and *Il17-GFP* mice were stimulated 3h *in vitro* with IL-6+IL-23 prior to intravenous injection into *Cd18*$^{-/-}$ mice, which were then infected with RFP *Aspergillus* and examined as described above. *Cd18*$^{-/-}$ mice given IL-6+IL-23 – stimulated neutrophils had significantly less RFP *Aspergillus* than mice given unstimulated neutrophils (Fig. 7e,f), even though there was no significant difference in total neutrophils in infected corneas (Fig. 7g). No neutrophils were detected in *Cd18*$^{-/-}$ corneas in mice not given donor neutrophils (Supplementary Fig. 6c). Further, *Cd18*$^{-/-}$ mice given IL-6+IL-23 – stimulated neutrophils from *Il17*$^{-/-}$, *Il17rc*$^{-/-}$, *Rorc*$^{GFP/GFP}$ or *Cybb*$^{-/-}$ mice had significantly more RFP *Aspergillus* than those given neutrophils from C57BL/6 mice or heterozygous littermates (Fig. 7h,i). As there was no difference in total neutrophils in infected corneas (Fig. 7j), these data implicate IL-17 – IL-17 receptor interactions in enhanced fungal killing *in vivo*, and show that hyphal growth is dependent on ROS produced by NADPH oxidase.

Discussion

In the current study, we present data showing that a sub-population of human and murine neutrophils constitutively express cytoplasmic RORγt in addition to IL-6 and IL-23 receptors, and rapidly produce IL-17 and express functional IL-17 receptors after IL-6 and IL-23 stimulation. Elevated IL-17 gene expression was previously reported in fungal keratitis patients, where neutrophils were found to be the predominant cells in the cornea [14]; however, IL-17 producing neutrophils are also present in human psoriatic and cutaneous T cell lymphoma lesions [9, 30]. Murine models as diverse as LPS – induced lung inflammation [10, 11], acute kidney ischemia – reperfusion injury [12], inhalation anthrax [31], early stage arthritis [32], pulmonary aspergillosis [13] and systemic histoplasmosis [33] also report neutrophils as a source of IL-17. Further, in contrast to neutrophils from psoriasis patients, which produce IL-17 after cell death and extracellular trap formation [9], our studies show that IL-17 producing neutrophils in infected corneas are 95% viable.

Although IL-23 has been implicated in IL-17 production by neutrophils [12, 13, 33], results from the current study demonstrate that IL-6 is required for IL-17 production *in vivo*. IL-6 and IL-23, but not IL-1β or TGF-β, are essential for IL-17 gene expression in murine and human neutrophils. These cytokine requirements are similar to natural killer cells [34], but are distinct from other innate lymphoid cells, including invariant NKT cells that require IL-1

and IL-23 [35], and γδ T cells, which require IL-23 and IL-1 or IL-18 to produce IL-17, but not IL-6 [36, 37]. However, given that the concentration of recombinant IL-6 and IL-23 required to induce IL-17 expression is higher than we detected in the serum of primed mice or in AspHE stimulated splenocytes, it is possible that additional cytokines can mediate neutrophil IL-17 production.

RORγt is primarily expressed in cells of the lymphoid lineage [8, 15, 16, 38]; however, using transgenic mice that express GFP under control of the *Rorc* promoter, we demonstrated constitutive GFP expression in neutrophils, and showed that RORγt is resident in the cytoplasm in unstimulated murine and human neutrophils. We also found that RORγt translocates to the nucleus following IL-6 + IL-23 stimulation, and can bind to oligonucleotides corresponding to the sequence of the IL-17 promoter. The presence of RORγt in the cytoplasm is distinct from Th17 cells, where RORγt is resident in the nucleus [39].

In addition to IL-17 production, results from the current study show that IL-6 and IL-23 also stimulate expression of the IL-17RC subunit of the receptor. As neutrophils constitutively express IL-17RA, IL-6 + IL-23 therefore induce expression of a functional IL-17 receptor on these cells. Other studies reported that unstimulated or LPS – stimulated neutrophils do not express IL-17RC and do not respond to rIL-17; however, they did not incubate the cells with IL-6 or IL-23 [22, 23]. IL-17RC expression was further elevated in the presence of fungal antigens, which was mediated by Dectin-2.

The c-type lectins Dectin-1 and Dectin-2 recognize fungal cell wall β-glucan and α-mannose, respectively [25], and Dectin-2 activation of dendritic cells plays an important role in development of Th17 responses [24, 40]. However, although Dectin-1 is expressed on unstimulated murine and human neutrophils ([26, 41-43] and the current study), we show that Dectin-2 is expressed on neutrophils only after stimulation with IL-6+IL-23. Low level expression of Dectin-2 on neutrophils was reported in a model of zymosan – induced inflammation [43], indicating that either IL-6 and IL-23 were produced in this model, or that other cytokines can stimulate Dectin-2 expression. Further, although Dectin-1 is activated by *Aspergillus* [44, 45], we found that Dectin-2 induces IL-17RC expression on neutrophils in the presence of these fungi.

Activation of IL-17 receptors on epithelial cells and fibroblasts stimulates production of pro-inflammatory and chemotactic cytokines that mediate recruitment of neutrophils to infected or inflamed tissues[1]. However, infiltration of this neutrophil population is likely to exacerbate the inflammatory response by IL-17 – IL-17 receptor mediated production of highly reactive oxygen radicals that not only have anti-microbial activity, but likely also have cytotoxic and tissue destructive activity.

Neutrophil subsets have been identified on the basis of nuclear morphology, granule protein content, cytokine production and receptor expression. For example, three neutrophil subsets are induced during infection with methicillin resistant *Staphylococcus aureus*, with each subset differing in morphology, cytokine production and anti-bacterial activity [46]. Also, a TGF-β responsive subset of neutrophils has pro-tumor activity in mice, which can be

reversed by anti-TGF-β [47]. A subset of human neutrophils expressing Mac-1 and ROS were found to inhibit T cell proliferation [48], and a subset of human neutrophils expressing the granule protein olfactomedin 4 in inflamed joints [49].

Results from the current study have identified a novel subset of human and murine neutrophils that constitutively express IL-6 and IL-23 receptors on the cell surface in addition to cytoplasmic RORγt, and which can produce IL-17 following stimulation. Further, this subset has been reported in a broad range of infectious and inflammatory conditions, indicating that regulating production and activity of these cells could have implications for future therapies.

Materials and Methods

Aspergillus strains and AspHE preparation

Aspergillus fumigatus strain Af-BP is a clinical isolate from a fungal keratitis patient treated at Bascom Palmer Eye Institute (Miami, FL) and used in our earlier studies [26]. The RFP expressing strain of *A. fumigatus* (Af-dsRed) is *gpd*A promoter driven to constitutively express monomeric dsRed [42]. *A.fumigatus* strain Af-BP hyphae were pulverized in liquid nitrogen, filtered through a 30 μm pre-separation filter (Miltenyi Biotec) and protein was measured using the BCA assay (Pierce). *Aspergillus* hyphal extracts (AspHE) were stored at -20°C and used at a final concentration of 1 mg/ml.

Source and maintenance of mice

The following mice were obtained from the Jackson Laboratories: C57BL/6J, $IL6^{-/-}$ (#002650 B6.129S2-Il6 tm1Kopf/J), $Cd18^{-/-}$ (#003991 B6.129S4-Itgam tm1Myd/J), $Rorc^{+/GFP}$ (RORγt reporter) and $Rorc^{GFP/GFP}$ (RORγt deficient) (#007572-B6.129P2(Cg)-Rorc.tm2Litt./J), and *Il17*- GFP reporter mice (#018472 C57BL/6-IL17atm1Bcgen/J). These transgenic mice from the Jackson laboratories used in the current study are on a C57BL/6J background, and C57BL/6J mice were used as controls. $Rag2^{-/-}Il2rg^{-/-}$ (#017707 Rag2 tm1.1Flv Il2rgtm1.1Flv Tg (SIRPA)1Flv/J) and the chronic granulomatous disease, CGD, $Cybb^{-/-}$) were also purchased from the Jackson Laboratories.

$Il17rc^{+/-}$ and $Il17rc^{-/-}$ mice were obtained from W. Ouyang (Genentech, San Francisco, CA) under a material transfer agreement with Xiaoxia Li. The $Il17a^{-/-}$, Dectin-1 deficient $Clec7a^{-/-}$ and Dectin-2 deficient $Clec4n^{-/-}$ mice were obtained from Yoichiro Iwakura (University of Tokyo, Japan) under a material transfer agreement with EP. All transgenic mice are on a C57BL/6 background and C57BL/6J mice or heterozygote littermates were used as controls. All animal protocols were approved by the Case Western Reserve University IACUC.

Isolation of murine bone marrow neutrophils

Total bone marrow cells were recovered from the femurs and tibias by flushing with RPMI and an 18 gauge needle, erythrocytes were lysed using commercial lysis buffer (eBioscience), and bone marrow cells were separated by density centrifugation using a discontinuous Percoll gradient (52%, 69%, and 78%) (Fisher). Cells at the 69% / 78%

interface were harvested, and neutrophil purity (>98%) was confirmed by flow cytometry and Wright-Giemsa staining.

Human neutrophils and peripheral blood mononuclear cell (PBMC)

Informed consent was obtained in accordance with the Declaration of Helsinki and the Institutional Review Board of the University Hospitals of Cleveland. Human neutrophils were isolated from the peripheral blood of healthy donors in 3% Dextran, and were isolated by Ficoll gradient centrifugation (Fisher Scientific). Neutrophil purity was assessed by Wright-Giemsa stain. Total peripheral white blood cells were separated by Ficoll centrifugation as described above, and the PBMC layer was washed and 1×10^6 cells were incubated 18h with 1mg/ml AspHE.

Subcutaneous injection (priming) with and preparation of splenocyte supernatants

Live, plate-grown *A.fumigatus* conidia were harvested and incubated 6h in Sabouraud dextrose broth to allow germination and expression of β-glucan, which initiates the host response [42]. Heat-killed, swollen (germinated) conidia (3×10^8/100 μl), were injected subcutaneously at the base of the tail. After 3 days, serum was collected by retro-orbital bleeding, mice were euthanized by CO_2 asphyxiation, and spleens were removed. A single cell suspension was prepared, red blood cells were lysed as before, and 1×10^6 spleen cells were incubated 18h with 1 mg/ml AspHE.

ELISA

Cytokines were quantified by 2-site ELISA according to the manufacturer's directions (R&D Biosciences).

In vitro activation of murine and human neutrophils

Murine and human neutrophils were suspended at 1×10^6 cells/ml, were incubated in 1ml of AspHE stimulated splenocyte or PBMC supernatants, or 20 μg/ml of recombinant IL-6 and 2 μg/ml IL-23 at 37°C / 5% CO_2. For cytokine neutralization studies, 20 μg/ml (final concentration) of anti-mouse or anti-human IL-6, IL-23, IL-1β, or TGF-β antibody was added to splenocyte or PBMC supernatants 2 hours prior to neutrophil incubation.

Flow cytometry

Antibodies used for flow cytometry are listed in Supplementary Table 2. Total bone marrow cells or isolated neutrophils were incubated 15 min with anti- mouse CD16/32 antibody (Fc block, clone 93 eBioscience) or human Fc receptor binding inhibitor (eBioscience), washed and incubated 20 min on ice with antibodies for cell surface markers. For intracellular staining, cells were incubated 20 min in IC Fixation buffer (eBioscience), followed by permeabilization buffer (eBioscience) and 1h incubation with antibody in the presence of Fc block. Cells were analyzed using a BD FACS Aria flow cytometer (also used for cell-sorting), an Amnis Image Stream flow cytometer, or a C6 Accuri flow cytometer (BD). All gates were set based on isotype controls.

Confocal imaging

Images were collected using UltraVIEW VoX spinning disk confocal system (Perkin Elmer, Waltham, MA) mounted on a Leica DMI6000B microscope equipped with HCX PL APO 100X/1.4 oil immersion objective using a 0.2 μm step size. Images were then imported into Metamorph Image Analysis Software (Molecular Devices Corp., Downington, PA) where maximum projections were generated from the original stacks and subjected to 'no neighbors' 2D deconvolution.

Quantitative PCR

Neutrophils RNA was extracted using the RNeasy mini kit according to the manufacturer's directions (Qiagen, Valencia, CA). The quality of RNA was checked by spectrophotometry, and only samples with a 260/280 ratio of 2.0 were used to generate cDNA. The SuperScript First Stand synthesis system (Invitrogen) was used to generate cDNA according to Manufacturers directions. Primers used in the current study are listed in Supplementary Table 3. We used the SYBR green system (Applied Biosystems, Carlsbad, CA) for quantitative PCR using ABI Biosystems. CT scores of q-PCR are listed in Supplementary Table 3. PCR products were then run on a 1% agarose gel, and visualized using Ethidium bromide.

Western blot of cell lysates and nuclear extracts

Neutrophils were incubated with supernatants of primed or naïve C57BL/6 splenocytes, or recombinant IL-6, IL-23, or both for 0, 15, 30, and 60 minutes. After treatment, cells were washed in PBS and lysed using ice-cold 1x lysis buffer (Cell Signaling Technology, Beverly, MA). Total protein was quantified using standard BCA assay, denatured with 2x Laemmli buffer (Sigma) and heated to 95°C for 5 minutes. Nuclear extracts of the cells were prepared using Pierce nuclear extract kit as per manufacturer's directions (Pierce). 10μg of total protein was analyzed in 12% SDS polyacrylamide gel, and transferred to nitrocellulose. Blots were probed with the following primary antibodies: RORγt (eBioscience), TATA box binding protein (TBP (Abcam), and β-actin (Cell Signaling Technology). HRP-conjugated secondary antibodies were used (Santa Cruz Biotechnologies), and blots were developed with Supersignal West Femto Maximum Sensitivity Substrate (Pierce).

Electrophoretic Mobility Shift Assay (EMSA)

A double stranded-biotin labeled oligonucleotide was synthesized based on DNA sequence present in the mouse IL-17A promoter region containing a putative RORγt binding site (underlined) was designed (sense: 5′-biotin-CTGTGCTGACCTCATTTGAGG-3′; antisense: 5′-biotin CCTCAAA TGAGGTCAGCACAG-3′). This sequence was derived from ChIP analysis of transfected HEK293 cells as described [19]. As a negative control, we designed an oligonucleotide that has three base substitutions in the binding region (sense 5′-biotin-CTGTGCT**T**A**T**C**G** CATTTGAGG-3′; antisense 5′-biotin-CCTCAAATG**C**G**A**T**A**AGCACAG-3′). Nuclear extracts (6 μg of protein) generated from bone marrow neutrophils were incubated with poly (dI-dC) and wild type biotinylated probes for 30 minutes at room temperature.

For competition assays, nuclear extracts were pre-incubated with non-labeled wild type probes for 10 minutes. For supershift assays, nuclear extracts were pre-incubated with anti-RORγt polyclonal antibody for 1 hour on ice. The samples were analyzed by 5% non-denaturing polyacrylamide gel electrophoresis followed by transfer to Biodyne B nylon membranes (PALL Life Science), and visualized using Supersignal West Femto Maximum Sensitivity substrate (Pierce).

In vitro fungal killing assay

A neutrophil-hyphae co-incubation assay was used to study the ability of murine and human neutrophils to inhibit *A.fumigatus* hyphal growth as described [26]. Briefly, 12,500 *A.fumigatus* dsRed expressing conidia (strain Af-dsRed) in 200 μl Sabouraud dextrose media were added to wells of black-walled 96 well plates with an optically clear bottom (CoStar 3720). After 6h, the conidia had germinated and the hyphae were adherent to the wells, and media was removed.

Murine and human neutrophils were stimulated 3h with IL-6+IL-23, washed, and 2×10^5 bone marrow derived murine neutrophils or 1×10^5 human peripheral blood neutrophils were added to each well with growing hyphae. RPMI media was used as positive control (unimpaired growth), and PBS was used as a negative control (no growth). After 16h incubation, wells were washed, and dsRed as a measure of fungal mass was quantified using a 96-well fluorometer at 550/600nm excitation/emission filter (Synergy HT, Biotek).

Murine models of fungal keratitis

Details of the adoptive transfer model were recently described [26]. Briefly, male and female $CD18^{-/-}$ mice aged 4-8 weeks were infected intrastromally with 20,000 live RFP expressing *A.fumigatus* (strain Af-dsRed) conidia. After 3h, 2×10^6 naïve or IL-6+IL-23 stimulated donor bone marrow neutrophils were injected into the tail vein of *Cd18*$^{-/-}$ recipient mice. In a second murine model, C57BL/6 and IL-17/GFP reporter mice were primed for three days as described above, and infected with the same number of live conidia. Twenty-four hours post infection, infected mice were euthanized and fungal growth in the cornea was imaged by fluorescent stereoscope and quantified by Metamorph software. Infected corneas were also collected, digested in collaganese, and cells were incubated with NIMP-R14 to quantify neutrophils in the cornea. A sample size of 3-5 mice per group was determined based on our past experience in generating statistical significance. Mice were randomized, and the only exclusion criterion was if they had obvious pre-existing corneal or conjunctival inflammation. Investigators were not masked for any of these studies.

Detection of ROS production by CFDA

In vitro ROS production measured by CFDA has been described [26]. Briefly, for ROS analysis, *A.fumigatus* conidia (Af-BP, 100,0000 /200μl) were incubated 6 h in SDB to germinate fungal hyphae as described above. 2×10^5 murine or human neutrophils were pulsed 10 min with 1μM H_2CFDA (Carboxyfluorescein diacetate, Sigma), which fluoresces after ROS oxidation, incubated 1h in wells with growing hyphae as described [26]. Neutrophils were collected from the wells, and analyzed by flow cytometry. H_2CFDA

pulsed neutrophils not incubated with fungal hyphae were used to set the ROS histogram gate. The average mean intensity was calculated by Accuri flow software.

Statistical analysis

Statistical analysis was performed for each experiment using an unpaired t-test and one-way ANOVA analysis with Tukey's post-hoc analysis (Prism, GraphPad Software). A p-value <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M.-J. Chang and G. Yakubenko for outstanding technical assistance. This work was supported by NIH grants RO1 EY018612 (EP), P30 EY011373 (EP), F32 EY007157 (PT), F31 EY019841 (SML), R01GM082916 (BAC), R01NS071996 (XL), and by the Research to Prevent Blindness Foundation and the Ohio Lions Eye Research Foundation.

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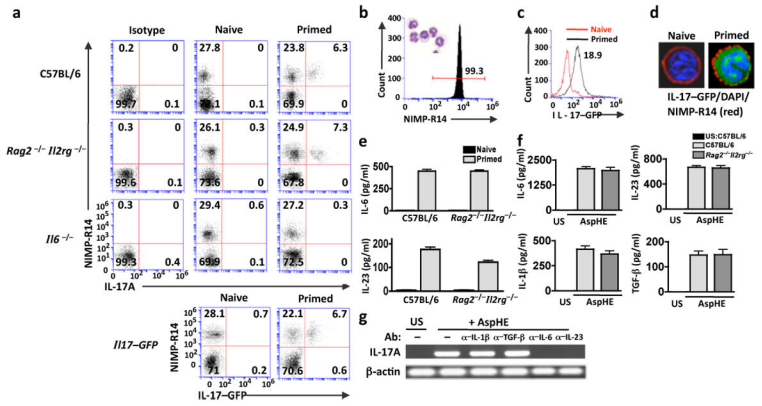

Figure 1. Induction of IL-17A producing neutrophils *in vivo*

(a) expression of intracellular IL-17A in NIMP-R14^{+} bone marrow cells from C57BL/6, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$, *Il6*$^{-/-}$ and *Il17a-GFP* reporter mice recovered three days after receiving a subcutaneous injection of heat-killed, swollen *Aspergillus fumigatus* conidia **(primed)**. **(b)** NIMP-R14^{+} neutrophils isolated from total bone marrow cells by density centrifugation showing purity of isolation (inset shows Wrights-Giemsa stained neutrophils). **(c)** Representative histogram showing percent IL-17A/GFP expressing NIMP-R14^{+} bone marrow neutrophils from naïve and primed *Il17a-GFP* mice. **(d).** Representative IL-17/GFP expressing cells from primed *Il17a-GFP* reporter mice (original magnification is ×400). **(e)** Serum IL-6 and IL-23 from naïve and primed C57BL/6 and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. **(f)** Cytokine production by splenocytes from naive C57BL/6 and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice following 18h incubation with *Aspergillus* hyphal extract (**AspHE**) or unstimulated (**US**). **(g)** IL-17A gene expression in naïve C57BL/6 bone marrow neutrophils incubated with media alone (unstimulated, **US**) or after 1h incubation with supernatants from AspHE – stimulated splenocytes plus neutralizing antibodies to IL-1β, TGFβ, IL-6 or IL-23. **(a-c)**: Representative scatter plots from four mice per group; **(e, f)**: mean +/- SD of four mice per group, $p<0.001$ naïve and primed mice **(e)**, and between unstimulated and AspHE – stimulated neutrophils **(f)**. Each experiment was repeated twice with similar results.

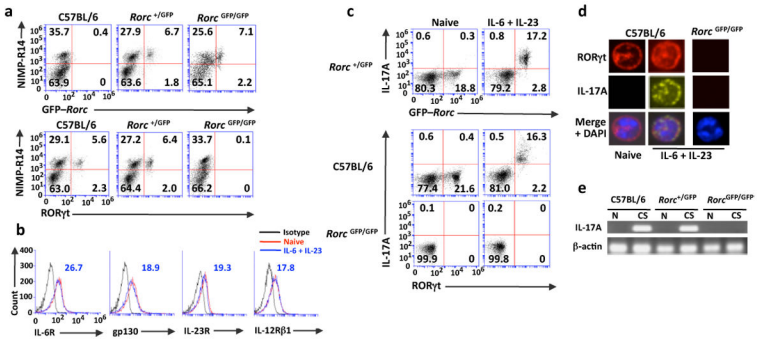

Figure 2. RORγt dependent IL-17 expression by neutrophils

(a) GFP-*Rorc*$^{+}$ NIMP-R14^{+} cells in total bone marrow cells from *Rorc*$^{+/GFP}$, and *Rorc*$^{GFP/GFP}$ mice (upper panels), and RORγt protein in NIMP-R14^{+} cells from C57BL/6 and *Rorc*$^{+/GFP}$ mice (lower panels). **(b)** IL-6R, gp130, IL-23R, and IL-12Rβ1 expression in naïve and IL-6+IL-23 – stimulated bone marrow neutrophils from C57BL/6 mice. **(c)** Intracellular IL-17A and GFP–*Rorc*$^{+}$ expression in IL-6+IL-23 – stimulated neutrophils (upper panels), and intracellular IL-17A and RORγt in bone marrow neutrophils from C57BL/6 and *Rorc*$^{+/GFP}$ mice. **(d)** Representative Confocal images showing expression of RORγt and IL-17 (original magnification is ×400). **(e)** IL-17 gene expression in IL-6+IL-23 stimulated neutrophils from C57BL/6 and *Rorc*$^{+/GFP}$ mice. 20μg of IL-6 and 2μg of IL-23 were used to stimulate all neutrophils. All data are representative of 4 mice per group and two repeat experiments.

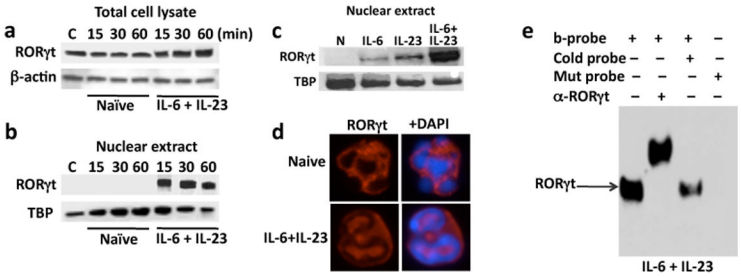

Figure 3. RORγt translocation to the nucleus of IL-6+IL-23 – stimulated neutrophils

Western blot of total cell lysates **(a)** and nuclear extracts **(b)** of isolated bone marrow neutrophils from naïve C57BL/6 mice after stimulation with supernatants from AspHE stimulated splenocytes containing IL-6+IL-23. **(c)** Nuclear extracts of neutrophils stimulated with 20μg recombinant murine (r)IL-6 and/or 2μg rIL-23. Blots were probed with antibody to RORγt, β-actin and the TATA box Binding Protein (TBP). **(d)** Representative Confocal images of intracellular RORγt in IL-6+IL-23 – stimulated C57BL/6 neutrophils and counterstained with DAPI. **(e)** Electrophoretic mobility shift assay (EMSA) analysis of IL-6+IL-23 stimulated bone marrow neutrophils from C57BL/6 mice. Nuclear extracts incubated with biotinylated oligonucleotide probes corresponding to the putative RORγt binding site of the mouse IL-17A promoter region. Extracts were also incubated with a biotinylated mutated (**Mut)** probe, with the biotinylated probe and competing cold probe, and with polyclonal anti- RORγt antibody (supershift assay). These experiments were repeated twice with similar results.

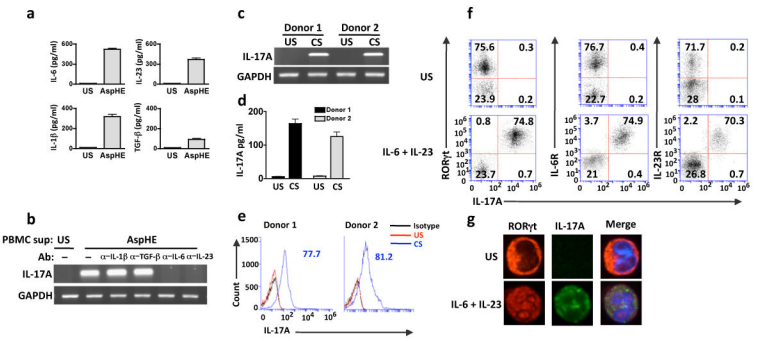

Figure 4. RORγt and IL-17A expression in human peripheral blood neutrophils

(a) Total cytokines in culture supernatants of peripheral blood mononuclear cells **(PBMC)** incubated 18h with AspHE (mean+/- SD). **(b)** IL-17 gene expression in neutrophils incubated 1h with PBMC supernatants (after stimulation with AspHE) plus neutralizing antibodies to IL-1β, TGF–β, IL-6 or IL-23. **(c-e):** IL-17 gene expression **(c)** total cellular IL-17 protein **(d)** and intracellular IL-17A **(e)** in IL-6+IL-23 – stimulated peripheral blood neutrophils from two healthy donors. **(f)** Intracellular IL-17 and RORγt, and cell surface IL-6R and IL-23R expression in unstimulated (upper panels) and IL-6+IL-23 - stimulated (lower panels) peripheral blood neutrophils from a single donor. **(g)** Confocal images of intracellular IL-17A and RORγt in neutrophils from a single donor. Neutrophils were stimulated with 20μg rIL-6 and 2μg rIL-23, and experiments were repeated using neutrophils from different donors.

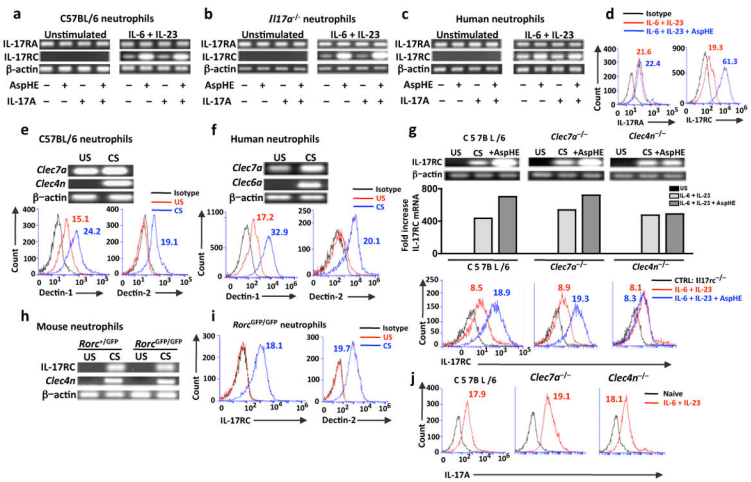

Figure 5. IL-17RA, IL-17RC and Dectin-2 expression in murine and human neutrophils (a-c)

IL-17RA and IL-17RC gene expression in bone marrow derived neutrophils from C57BL/6 mice **(a)**, IL-17-/- mice (**b)**, and human peripheral blood **(c)** after 1h incubation with IL-6+IL-23, and a further 1h with AspHE. **(d)** Cell surface IL-17RA and IL-17RC on IL-6+IL-23 stimulated human neutrophils. **(e)** *Clec7A* (Dectin-1) and *Clec4n* (murine Dectin-2) gene and cell surface expression in IL-6+IL-23 – stimulated C57BL/6 bone marrow neutrophils. **(f)** *Clec6a* (human Dectin-2) and *Clec7A* gene and cell surface expression in IL-6+IL-23 – stimulated human peripheral blood neutrophils. **(g)** IL-17RC expression in IL-6+IL-23 stimulated bone marrow neutrophils from C57BL/6, *Clec7a*$^{-/-}$ and *Clec4n*$^{-/-}$ mice after incubation with AspHE. Neutrophils from *Il17rc*$^{-/-}$ mice were used as negative controls (CTRL). **(h)** IL-17RC and *Clec4n* gene expression in IL-6+IL-23 – stimulated bone marrow neutrophils from *Rorc*$^{+/GFP}$ and *Rorc*$^{GFP/GFP}$ mice. **(i)** IL-17RC and Dectin-2 cell surface expression in IL-6+IL-23 – stimulated bone marrow neutrophils from *Rorc*$^{GFP/GFP}$ mice. **(j)** Intracellular IL-17A in IL-6+IL-23 – stimulated neutrophils from *Clec7a*$^{-/-}$ and *Clec4n*$^{-/-}$ mice. Data with mouse cells are representative of four mice, and experiments were repeated twice with similar results. Data with human cells are from a single donor, with a repeat experiment; results from additional donors are shown in Supplemental Figure S4.

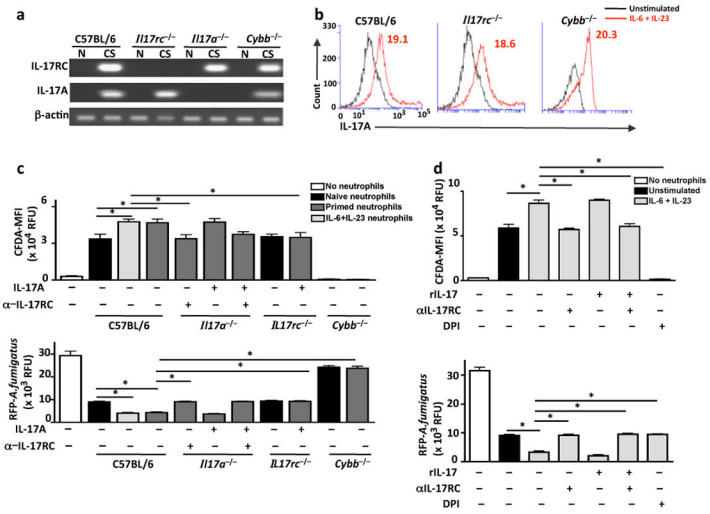

Figure 6. The role of IL-17A and IL-17RC in production of neutrophil reactive oxygen species (ROS) and hyphal growth *in vitro*

IL-17RC and IL-17A gene expression **(a)** and intracellular IL-17A **(b)** in IL-6+IL-23 – stimulated neutrophils from *Il17a*$^{-/-}$, *Il17rc*$^{-/-}$, and *Cybb*$^{-/-}$ mice (data are representative of three mice per group). (**c, upper panel**) ROS production (intracellular CFDA) in bone marrow neutrophils from naïve or primed C57BL/6 mice, and from naïve *Il17a*$^{-/-}$, *Il17rc*$^{-/-}$, and *Cybb*$^{-/-}$ mice stimulated 3h with IL-6+IL-23 and then incubated 1h with growing *Aspergillus* hyphae +/- rmIL-17A or anti-IL-17RC. **RFU**: Relative fluorescent units; **(lower panel)** Fungal growth of dsRed expressing *Aspergillus* after 18h incubation with each neutrophil population. Fungal mass was measured by fluorimetry of dsRed, and represented as RFU. Mean +/- SD of three samples per group; controls are medium only **(Ctrl)** and naïve neutrophils (**naïve**). **(d)** ROS production and fungal mass of human peripheral blood neutrophils incubated with *Aspergillus* hyphae +/- rhIL-17A, anti-IL-17RC, or the ROS inhibitor DPI. **(c-f)** mean +/- SD of three wells per experimental condition from neutrophils pooled from three mice per group **(c)** or from a single donor **(d)**. Data are representative of three repeat experiments.

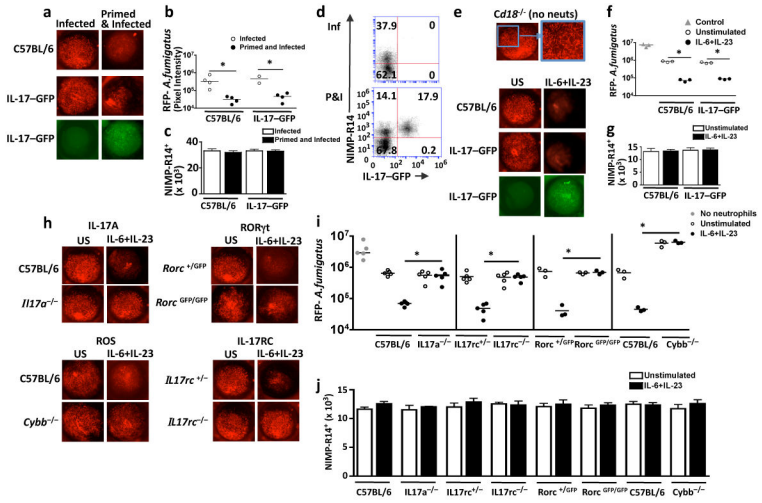

Figure 7. The role of neutrophil IL-17, IL-17RC and NADPH oxidase in regulating RFP-*Aspergillus* hyphal growth *in vivo* – (a-d)

Primed *Il17*–GFP reporter mice infected with RFP *Aspergillus* showing representative corneas 24h post-infection **(a)**, total RFP in infected corneas assessed by image analysis **(b)** (data points represent individual corneas), total NIMP- R14^{+} neutrophils **(c)**, and percent IL-17/GFP expressing neutrophils in the corneal stroma **(d)**. **(e-j)** IL-6+IL-23 – stimulated bone marrow neutrophils from *Il17*–GFP, *Il17a*$^{-/-}$, *Il17rc*$^{-/-}$, and *Cybb*$^{-/-}$ mice stimulated were injected intravenously into recipient *Cd18*-/- mice, which were then infected intrastromally with RFP *Aspergillus.* Representative corneas of *Il17*–GFP 24h post-infection (**e**), total RFP in infected corneas assessed by image analysis (**f**) (data points represent individual corneas), and total NIMP- R14^{+} neutrophils **(g)**. Representative images of *Aspergillus* infected *Cd18*$^{-/-}$ corneas in mice given *Il17a*$^{-/-}$, *Il17rc*$^{-/-}$, or *Cybb*$^{-/-}$ neutrophils **(h)**, total RFP *Aspergillus* **(i)**, and total neutrophils **(j)**. Experiments were repeated twice with similar results.