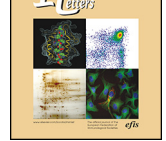




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A lack of confirmation with alternative assays questions the validity of IL-17A expression in human neutrophils using immunohistochemistry

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ABSTRACT

We identified IL-17A-positive neutrophils in *Wolbachia*-positive *Onchocerca volvulus* nodules using an antibody that has previously reported IL-17A-positive neutrophils in several inflammatory conditions. However, we could not detect IL-17A using a range of alternative assays. Our data question the IL-17A antibody specificity and the ability of human neutrophils to express IL-17A.

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1. Introduction

Interleukin-17 (IL-17) is an important mediator of inflammatory and infectious disease [1] and the main source of IL-17 has, until recently, been believed to be Th17 cells [2]. While a number of recent reports have proposed that neutrophils are a source of IL-17 in inflammatory and infectious diseases in both mouse and humans [3–20], the ability of human neutrophils to express IL-17A is controversial [21].

The pathologic features of onchocerciasis mirror IL-17-mediated immune responses. The combination of stimuli from the filarial nematode and its bacterial endosymbiont *Wolbachia* could promote a cytokine milieu favourable for the induction of an IL-17-mediated immune response [22], but little is currently known about the actual presence and role of such a response in filariasis [23–25]. We identified IL-17A expressing neutrophils in *Wolbachia*-positive *Onchocerca volvulus* nodules from infected Cameroonian patients [26] using immunohistochemistry (IHC) staining with a commercially available antibody (R&D Systems, goat polyclonal anti-IL-17A,

AF-317-NA) that has been used in other studies of IL-17A expression in neutrophils [4–13]. However, when we tried to confirm this finding using alternative assays, we were unable to confirm this expression of IL-17 by human neutrophils.

2. Methods

2.1. Immunohistochemistry staining of *O. volvulus* nodules

Nodules from patients infected with *O. volvulus* were surgically removed, fixed in 80% ethanol and paraffin-embedded as part of the evaluation of a phase II trial of doxycycline [26]. Sections of 4 µm were cut by microtome and mounted in an electrothermal bath at 45 °C on poly L-lysine slides (which were incubated overnight at 58 °C before use). After re-hydration and antigen retrieval (performed in an 800 W microwave with two cycles of 3.5 min in 1 mM EDTA pH 8.0), endogenous peroxidase was quenched by incubation for 30 min in 3% H₂O₂ in methanol, before sections were blocked for 30 min in TNB blocking buffer. IHC staining was carried out using the following antibodies: goat anti-human IL-17A (1:10 or 1:50, AF-317-NA R&D Systems), mouse anti-human CD4 (1:40, 1-F6 Novocastra), donkey anti-goat IgG-HRP conjugated (1:500 or 1:1000, OB1500P AbD Serotec), goat anti-mouse IgG-HRP conjugated (1:800, NEF822 Perkin Elmer). The tyramine signal amplification (TSA) (FITC or Cy3, Perkin Elmer) system was used to detect antibody bound to the target. Slides stained omitting

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the primary antibody were included as a control for non-specific binding of the secondary antibody. The use of normal goat IgG in place of the primary antibody controlled for non-specific binding of the goat anti-human IL-17A IgG antibody. To confirm total quenching of endogenous peroxidase, TSA-Cy3 staining was carried out immediately after the H₂O₂ quenching step. No signal was detected, confirming that no endogenous peroxidase was present. DAPI was used to stain nuclear DNA. H&E staining was performed using standard techniques.

2.2. Isolation of neutrophils and PBMCs

Participants gave informed consent according to the Declaration of Helsinki. Ethics approval was obtained from the University of Liverpool Committee on Research Ethics and Liverpool School of Tropical Medicine Research Ethics Committee. Blood was collected into lithium-heparin vacutainers and separated into cellular fractions using Polymorphprep (Axis-Shield). PBMCs were isolated from the upper band and neutrophils from the lower band, as previously described [27]. Neutrophil purity was >97% and viability was >98%. For some experiments (as indicated in Section 3), neutrophils were separated from whole blood using the Easy-Sep Human Neutrophil Enrichment kit (Stem Cell), which provided negatively selected (untouched) neutrophils with 99.9% purity. Neutrophils and PBMCs were incubated at 37 °C in a humidified incubator in RPMI 1640 (with 25 mM HEPES and 2 mM L-glutamine) culture media (Gibco). Neutrophils and/or PBMCs were incubated for up to 15 h with synthetic WoLP (1 µg/mL) (the primary *Wolbachia*-derived pro-inflammatory molecule (EMC Microcollections) [28]), ultra-pure LPS (0.1 µg/mL, Sigma), DMSO (Sigma), or filarial extracts containing *Wolbachia* (BmFE, 200 µg/mL) and depleted from *Wolbachia* (BmFETet, 200 µg/mL). PBMCs were incubated for up to 15 h with PMA (50 ng/mL, Sigma)/ionomycin (10 µg/mL, Sigma) to induce differentiation of CD4⁺/IL-17⁺ T-cells.

2.3. Western blotting

Neutrophils and PBMCs were lysed in boiling Laemmli buffer. Protein samples (10⁶ cells) were separated by SDS-PAGE (12–14% gel) and transferred onto PVDF membrane (Millipore). Culture supernatant was precipitated in ice cold acetone (1:3 v/v) overnight at –20 °C, followed by centrifugation at 900 × g for 3 min. The pellet was lysed in 100 µL boiling Laemmli buffer, and 30 µL was loaded. Recombinant human IL-17A (R&D Systems) was used as a positive control (16.6 ng/lane). Antibodies used were goat anti-human IL-17A (AF-317-NA, R&D Systems, 1:500 or 1:2500), mouse anti-human IL-17A (41802, R&D Systems, 1:500 or 1:5000), rabbit anti-human IL-17A (PRS4877, Sigma, 1:500), goat anti-human IL-17A (sc-6077, Santa Cruz, 1:100), HRP-conjugated donkey anti-goat IgG (OBT1500P, AbD Serotec, 1:5000), HRP-conjugated goat anti-mouse IgG (NEF822, Perkin Elmer, 1:5000), HRP-conjugated goat anti-mouse IgG/IgM (M30907, Caltag; 1:50,000), and HRP-conjugated mouse anti-goat IgG (31400, Thermo Scientific; 1:100,000). Bound antibodies were detected using the ECL system (Millipore) or SuperSignal West Dura Extended Duration Substrate for HRP (Thermo Scientific) on carefully-exposed film (Amersham) to avoid saturation.

2.4. Measurement of IL-17A by ELISA and Luminex

Levels of IL-17A in cell lysates and culture supernatants were measured using two different ELISA kits (DuoSet ELISA Development kit, R&D Systems; ELISA Ready-SET-Go! Kit, eBiosciences) and two different Luminex kits (Bio-Plex Pro Reagent kit, Bio-Rad; Milliplex MAG Immunoassay kit, Millipore) as per the manufacturer's instructions. Absorbance readings from the ELISA assays

were measured using a FLUOstar Omega plate reader, and Luminex assays were analysed in a Bio-Plex system (Luminex xMAP technology). Minimum detection limits of IL-17A were 7.8 pg/mL for ELISA and 3.2 pg/mL for Luminex.

2.5. Isolation of RNA and analysis by RNA-Seq and RT-PCR

RNA was isolated using TRIzol-chloroform (Invitrogen) as previously described [27], and cleaned using an RNeasy mini kit (Qiagen), including a DNA digestion step. RNA-Seq analysis of neutrophil mRNA was carried out on the Illumina platform as previously described [27]. For RT-PCR, total RNA was reverse transcribed into cDNA using SuperScript III first strand synthesis system, as per the manufacturer's instructions. PCR primers were: IL-17A forward 5'-ACTACAACCGATCCACCTCAC-3', reverse 5'-ACTTTGCCTCCAGATCACAG-3'; ribosomal protein S12 (housekeeping gene) forward 5'-GAATTCGCGAAGCTGCCAAA-3', reverse 5'-GACTCCTTGCCATAGTCCTT-3'. Amplification was performed with Quantitect SYBR Green PCR kit, as per the manufacturer's instructions, using an annealing temperature of 55 °C and 40 cycles. PCR products were visualised on a 1% agarose gel. For the sequencing of PCR products, bands were excised and DNA extracted using QIAEX II gel extraction kit. Extracted DNA was sequenced using a Beckman Coulter Genomics system (Essex, UK).

2.6. Immunoprecipitation and mass spectrometry

PMN (5 × 10⁶ in 1 mL) and PBMC (1 × 10⁶ in 200 µL) were lysed in Pierce IP Lysis Buffer (Thermo Scientific) as per the manufacturer's instructions. Immunoprecipitation was carried out using 100 µg mouse anti-human IL-17A antibody (41802, R&D Systems) and 60 µg goat anti-human IL-17A antibody (AF-317-NA, R&D Systems) coupled to 1 mL HiTrap NHS-activated HP columns and AKTAPrime Plus low-pressure affinity chromatography system (both from GE Healthcare) using the manufacturer's protocol. Protein samples underwent chromatographic separation prior to mass spectrometric analysis using an UntimateTM3000 system. Electrospray ionisation tandem mass spectrometry (ESI-MS/MS) was performed using an LTQ Orbitrap Velos mass spectrometer. MS/MS data were processed using Proteome Discoverer 1.1TM software and searched against the non-redundant human database from Uniprot using the MASCOT search engine.

3. Results

IHC staining of *Onchocerca* nodules indicated strong IL-17A signals in cells around the *Wolbachia*-containing worms (Fig. 1A). In order to identify the cellular source of the IL-17A signal, we counterstained the nodules for CD4 and found that only 5.16% of CD4⁺ T-cells were IL-17A⁺, but that the majority of IL-17A⁺ cells (>70%) did not stain CD4⁺ (Fig. 1B). We used sequential H&E staining of sections stained for IL-17A to identify the IL-17A⁺ cells as neutrophils (Fig. 1C), and found that IL-17A⁺ neutrophils were localised within *Onchocerca* nodules in close proximity to *Wolbachia*-containing worms (Fig. 1D).

In order to further investigate IL-17A expression by human neutrophils in response to *Wolbachia*, we carried out IHC on donor-derived neutrophils following stimulation with WoLP. We found that 0 h healthy control neutrophils stained positive for IL-17A using this assay, and that expression of IL-17A was upregulated by stimulation with WoLP (Fig. 1E). In contrast, healthy control PBMCs only stained for IL-17A when cells were differentiated into CD4⁺/IL-17A⁺ Th17 cells (by stimulation with PMA/ionomycin, data not shown). Staining was independent of ethanol or formalin fixation (data not shown).

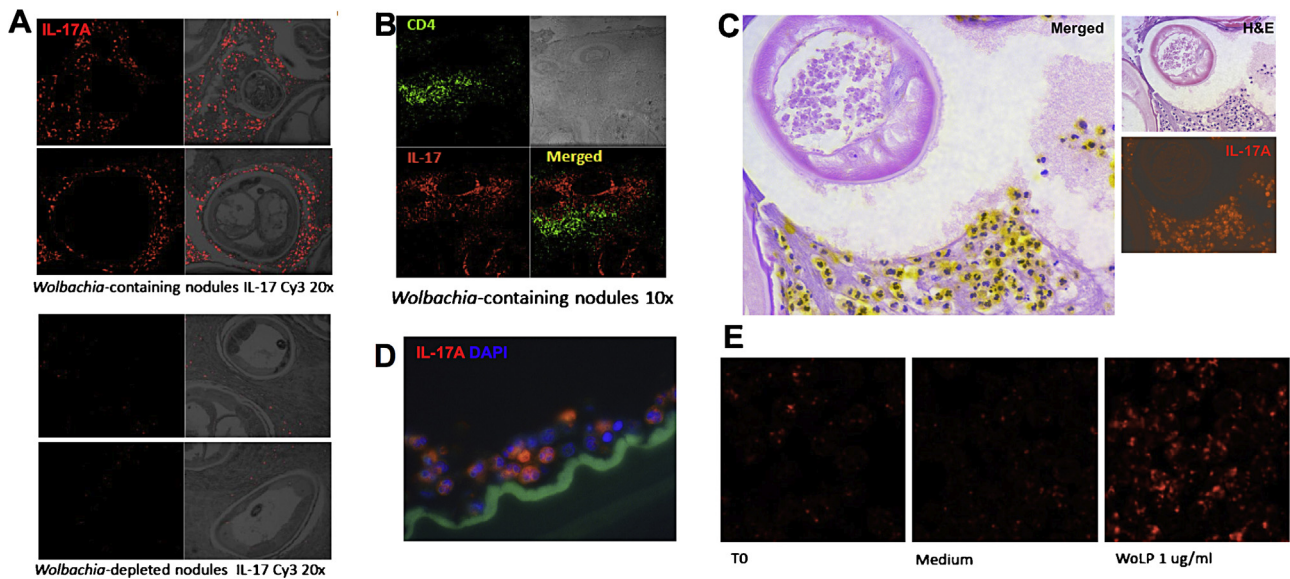


Fig. 1. Detection of IL-17A in human neutrophils by IHC. (A) Cyanine3 labelled IL-17A⁺ cells infiltrate *Wolbachia*-containing nodules but not *Wolbachia*-depleted nodules. (B) Counterstaining with FITC labelled CD4⁺ antibody revealed the IL-17A⁺ cells were not Th17 cells. (C) H&E staining identified the IL-17A⁺ cells as neutrophils. (D) Representative image of polymorphonucleated and mononucleated cells in *O. volvulus* nodule. Sections were stained for IL-17A (Cyanine3, red) and DNA (DAPI, blue). The *Wolbachia* worm membrane is shown in green. (E) IHC of healthy control neutrophils at time 0 h or treated for 4 h in the absence or presence of WoLP (1 µg/ml) revealed a high proportion of neutrophils which were IL-17A⁺ (Cyanine3, red). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

In order to investigate expression of IL-17A by neutrophils further, we carried out Western blot analysis of neutrophil lysates incubated for up to 12 h with WoLP, DMSO (WoLP vehicle control), LPS or in media alone (Fig. 2A). Western blotting using the same antibody used for IHC (AF-317-NA, R&D, goat) identified multiple bands in all samples, but none of the bands corresponded to the molecular weight of rh-IL-17A (lane 1, Fig. 2A). We therefore repeated our analysis with a second IL-17A antibody (41802, R&D,

mouse). Whilst this antibody only detected one band, the relative mobility did not correspond to the molecular mass of rh-IL-17A (Fig. 2A). We repeated our analysis using PBMCs incubated for up to 12 h with PMA/ionomycin, WoLP, DMSO, LPS or media alone. Western blotting using the IHC antibody (AF-317-NA, R&D, goat) did not detect any bands in any PBMC lysates, but did detect IL-17A in PMA/ionomycin PBMC culture supernatant (Fig. 2B). Only when membranes were processed with long-exposure ECL, a band

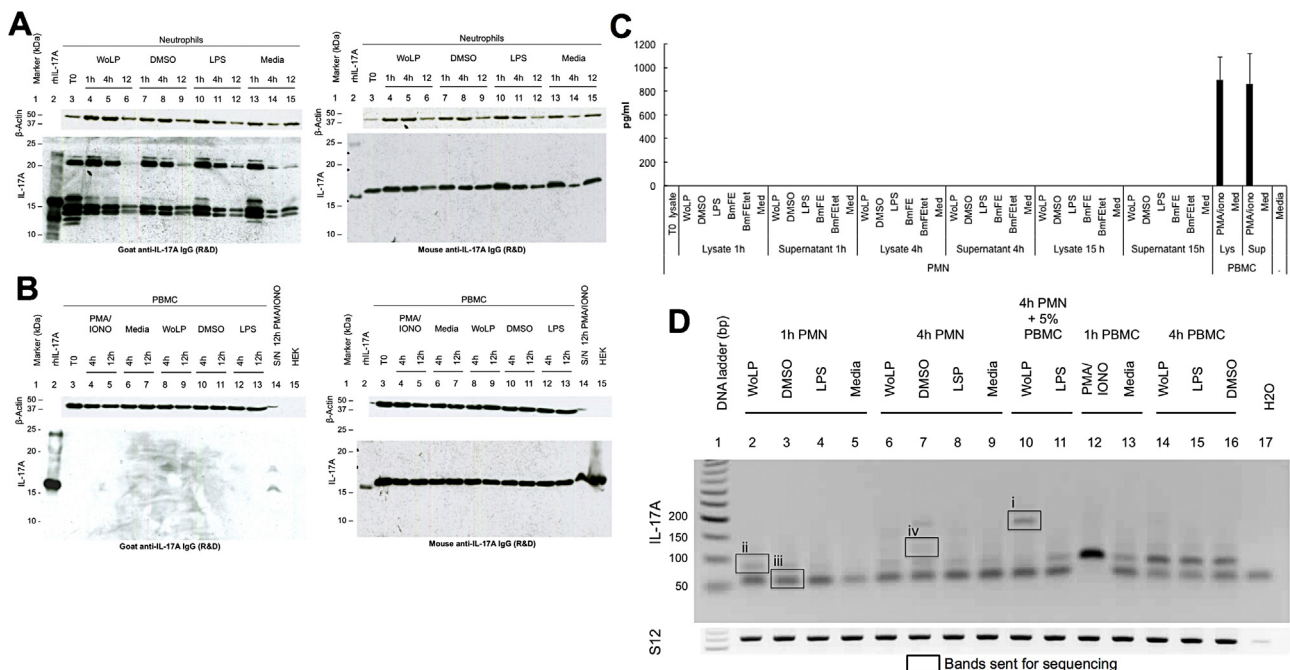


Fig. 2. Determination of IL-17A expression by human neutrophils. (A) Western blotting of neutrophil (PMN) cell lysate using goat anti-IL-17A antibody and mouse anti-IL-17A antibody. Bands detected were not of the same molecular weight as rhIL-17A (lane 1). (B) Goat anti-IL-17A antibody did not detect any protein in PBMC cell lysate but did detect rhIL-17A (lane 1). Mouse anti-IL-17A antibody detected a band in all samples (including HEK cell lysate, negative control), which did not correspond to the molecular weight of rhIL-17A (lane 1). (C) ELISA analysis of neutrophil and PBMC cell lysate and supernatant only detected IL-17A in PBMC samples incubated for 12 h with PMA/ionomycin (lysate and supernatant). (D) RT-PCR of PBMC mRNA detected IL-17A mRNA in PBMC treated for 1 h with PMA/ionomycin (lane 12). No band corresponding to IL-17A was detected in neutrophil mRNA. Four bands were excised and sequenced (details in text).

Table 1
Identification of proteins precipitated from neutrophil cell lysate following incubation for 4 h with WoLP (1 µg/mL) by goat anti-IL-17A and mouse anti-IL-17A antibodies (both R&D Systems).

IP antibody	Uniprot accession	Protein description	Protein accession	MASCOT score	Coverage (%)	No. of peptides
Goat-anti human IL-17A	P05164-2	Myeloperoxidase Isoform H14	PERM.HUMAN	556.76	12.00	9
	A5A6M6	Keratin, type II cytoskeletal 1	K2C1.PANTR	431.18	16.64	8
	H2Q7J2	Uncharacterised protein	H2Q7J2.PANTR	402.60	16.43	7
	P35908	Keratin, type II cytoskeletal 2	K22E.HUMAN	329.34	11.11	6
	B7Z4X2	Lactoferrin-C	B7Z4X2.HUMAN	301.00	9.01	4
	H2QCX3	Uncharacterised protein	H2QCX3.PANTR	222.35	6.41	3
	B3KSI4	Uncharacterised protein	B3KSI4.HUMAN	181.12	6.30	2
	P07737	Profilin-1	PROF1.HUMAN	171.92	28.57	3
	F8VV32	Lysozyme C	F8VV32.HUMAN	105.66	10.19	2
	Mouse anti-human IL-17A	P13645	Keratin, type I cytoskeletal 10	K1C10.HUMAN	918.76	35.95
P04264		Keratin, type II cytoskeletal 1	K2C1.HUMAN	880.01	27.95	16
P35908		Keratin, type II cytoskeletal 2	K22E.HUMAN	771.04	24.41	12
P59666		Neutrophil defensin	DEF3.HUMAN	702.51	19.15	2
P05164-2		Myeloperoxidase Isoform H14	PERM.HUMAN	682.52	18.46	13
P20160		Azurocidin	CAP7.HUMAN	355.58	21.91	5
P35527		Keratin, type I cytoskeletal 9	K1C9.HUMAN	347.65	9.47	4
B2MUD5		Neutrophil elastase	B2MUD5.HUMAN	124.32	9.38	2

of the same size was detectable in PBMC samples (data not shown). A second anti-IL-17A antibody (41802, R&D, mouse) detected a band in all samples at the same molecular mass as was detected in neutrophil lysates. However, this did not have the same electrophoretic mobility as rh-IL-17A, and was also detected in HEK cell lysate which was used as a negative control (Fig. 2B). Two further anti-human IL-17A antibodies were tested (sc-6077, Santa Cruz; PRS4877, Sigma). The Santa Cruz antibody gave multiple non-specific bands and the Sigma antibody gave results overlapping with the R&D (goat) antibody (data not shown).

We next measured IL-17A in cell lysates and culture supernatants using two ELISA (R&D kit, and eBioscience kit) and two Luminox (Bio-Rad kit, and Millipore kit). Results were consistent across all four independent kits. IL-17A was only detected in lysate and supernatant from PMA/ionomycin differentiated PBMCs. No IL-17A was detected in neutrophil lysates or supernatants after incubation under several experimental conditions (up to 15 h stimulation with WoLP, LPS, BmFE, BmFEtet or DMSO). Importantly, the capture antibody in the ELISA kit from R&D was the same antibody that gave a positive IHC result and a negative Western blot result (AF-317-NA, goat anti-human IL-17A). Representative data for the eBioscience ELISA is shown in Fig. 2C.

Analysis of mRNA by RT-PCR was carried out on neutrophils, PBMCs, and neutrophils containing a 5% PBMC "spike" after incubation for up to 4 h in the absence and presence of WoLP, DMSO, or LPS, and in PBMCs incubated for 1 h with PMA/ionomycin. Agarose gel analysis only identified a band of the correct size in the PMA/ionomycin PBMC sample (Fig. 2D). Multiple bands were detected in neutrophil samples. Four of these (labelled i–iv in gel) were excised from the gel and sequenced, and found to represent either [i] FAM50A gene (unrelated), [ii and iv] IL-17A, or [iii] primer dimers. The IL-17A bands in the neutrophil samples were the same intensity as in the 5% PBMC spiked sample, and it is likely that the contaminating PBMCs in the neutrophil samples are the source of IL-17.

Analysis of mRNA by RNA-Seq found no transcripts for IL-17A (RPKM=0) in neutrophils from RA patients ($n=29$), healthy controls ($n=7$) or healthy neutrophils treated for 1 h with a range of inflammatory cytokines (TNF, GM-CSF, G-CSF, IL-6, IL-1 β , IL-8, IFN α , IFN γ , $n \geq 3$). A comparison of neutrophils isolated by polymorphprep (97% pure) or negative-selection (>99.9% pure) also failed to identify any transcripts for IL-17A, or indeed any of the IL-17A-F cytokines (RPKM < 0.3).

Finally, we performed mass-spectrometry on cell-lysates from neutrophils stimulated for 4 h with WoLP and immunoprecipitated with both R&D antibodies (goat, mouse). The results

are shown in Table 1. IL-17A was not detected in either sample, while it was correctly identified in the rh-IL-17A control sample.

4. Discussion

Our investigation identified IL-17A positive neutrophils in *Wolbachia*-positive *O. volvulus* nodules from infected patients using a commercially-available antibody which has previously been used to identify IL-17A expression by neutrophils in other published studies [4–13]. However, further investigation using a comprehensive range of laboratory techniques failed to detect transcription of IL-17A mRNA, or expression and/or secretion of IL-17A protein by human neutrophils under different experimental conditions. Furthermore, several commercially available anti-human IL-17A antibodies were tested using Western blotting of neutrophil and PBMC protein lysates and were shown to detect non-specific bands that did not correspond to IL-17A. Importantly, IP using the antibody that gave a positive result for IHC failed to identify IL-17A in neutrophil cell lysates. This antibody detected multiple non-specific bands in neutrophil cell lysate by Western blot, and was the capture antibody in the R&D ELISA kit, which also failed to detect IL-17A in neutrophil cell lysates or culture supernatants. One possible way of testing the antigen-specificity of an antibody is by pre-absorbing it with the antigen and then assessing the negative staining of an antigen-positive sample in IHC when the pre-absorbed antibody is used. In the case of goat anti-human IL-17A (AF-317-NA, R&D Systems), this approach was successful in the work of Appel et al. [6], but in the experimental work presented here it resulted in non-specific background staining in all conditions tested (data not shown).

Since the completion of our experiments described here, Taylor et al. [14] have reported IL-17A expression by murine and human neutrophils. However, our experimental approach differed from theirs in a number of important aspects. First, in our experiments, Ct values from qPCR experiments in excess of 30 cycles generate unreliable data, especially when using RNA extracted from neutrophil preparations that are contaminated with other leukocytes or when the RNA extraction method fails to include a DNase step. We consistently find that qPCR experiments using neutrophil RNA that has not included a DNase step are inevitably contaminated with DNA that contributes to qPCR reactions. Second, we do not use cytokines at such high concentrations (20 µg/mL).

Our comprehensive study raises doubt over the ability of human neutrophils to express IL-17A and we suggest caution should be taken when interpreting IHC analysis of IL-17A-positive

neutrophils using commercially available antibodies in the absence of a positive IL-17 signal from other detection techniques.

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