The role of innate immune cells in tissue inflammation in spondyloarthritis

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HUMAN MAST CELLS CAPTURE, STORE, AND RELEASE BIOACTIVE EXOGENOUS IL-17A

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J leukoc biol. 2016;Sep;100(3):453-62
ABSTRACT

IL-17A, a major pro-inflammatory cytokine, can be produced by a variety of leukocytes, but its exact cellular source in human inflammatory diseases remains incompletely understood. IL-17A protein is abundantly found in mast cells in human tissues such as inflamed synovium, but surprisingly, mechanistic murine studies failed to demonstrate IL-17A production by mast cells. Here, we demonstrate that primary human tissue mast cells do not produce IL-17A themselves but actively capture exogenous IL-17A through receptor-mediated endocytosis. The exogenous IL-17A is stored in intracellular granules and can subsequently be released in a bioactive form. This novel mechanism confers to mast cells the capacity to steer IL-17A-mediated tissue inflammation by the rapid release of pre-formed cytokine.
INTRODUCTION

The discovery of the IL-23/IL-17 axis introduced a new paradigm in tissue inflammation and autoimmunity [1,2]. Originally, studies using IL-23p19-deficient mice demonstrated that IL-23 drives T cell-mediated tissue inflammation [3,4]. Follow-up studies identified T helper type 17 (T\textsubscript{H}17) cells producing the signature cytokine interleukin-17A (IL-17A) as a distinct T helper cell lineage which is highly responsive to IL-23R signaling and produces IL-17A, IL-17F, and IL-22 under control of retinoic acid receptor related orphan receptor γt (ROR\textgamma t) [5–7]. The IL-23/IL-17 pathway has been proposed to be the central driver of organ-specific inflammation in several immune-mediated inflammatory diseases. This concept has now been validated in humans by the clinical efficacy of blockade of p40 (the common subunit of IL-23 and IL-12) [8–10], IL-17A [11–13], or IL-17RA [14,15] in inflammatory diseases such as psoriasis, psoriatic arthritis, and ankylosing spondylitis. In contrast, IL-17A inhibition was ineffective in Crohn’s disease despite the genetic and clinical overlap with ankylosing spondylitis and psoriasis and despite the efficacy of IL-23p40 blockade in Crohn’s disease [16,17]. This suggests that the IL-23/IL-17 axis can operate in different ways in different tissues and that the functional outcome of intervention in this pathogenic axis is highly dependent on the immunological context [18].

Therefore, significant effort has been put by the scientific community in the identification of the cellular source of IL-17A in different human disorders. In peripheral blood of patient with IL-17A-dependent conditions such as ankylosing spondylitis, a fraction of T cells, including T\textsubscript{H}17 cells and γδ-T cells, appear to be the major producers of IL-17A upon ex vivo stimulation [19–21]. In sharp contrast, immunohistological studies of inflamed target tissues such as synovial membrane in rheumatoid arthritis and spondyloarthritis [22–26], skin from patients with psoriasis and systemic sclerosis [27–29], renal tissue from rejected allografts and ANCA-associated vasculitis patients [30,31], atherosclerotic plaques [32], ALS lesions [33], lymph node and spleen from patients with chronic lymphatic leukemia [34], and esophageal and gastric carcinoma samples [35,36] identified innate immune cells such as mast cells and neutrophils, but not T\textsubscript{H}17 cells, as major cell types stained positively for IL-17A protein. Moreover the numeric presence of IL-17\textsuperscript{+}-mast cells is different between diagnosis in skin [27] and in synovial tissue [37] and is a predictor of negative outcome in renal allografts [31] and worse survival in gastric cancer [36]. Interestingly, outside the tumor nest of esophageal squamous cell carcinoma, in the muscularis proria, an increased number of IL-17A\textsuperscript{+}-mast cells correlated with a better outcome [35]. These studies are not powered to dissect if IL-17A\textsuperscript{+}-mast cells are causative for the disease phenotype, but they do emphasize the importance of this new cell type for immunopathology.

The discovery of IL-17\textsuperscript{+}-mast cells in human tissues is intriguing since studies employing IL-17A, IL-17F, IL-23R, and ROR\textgamma t-reporter mice did not identify mast cells or neutrophils among IL-17 producing cells [38–42]. This discrepancy may point to functional differences in the IL-17A axis between human inflamed tissues and experimental animal models. The present study aims to investigate in detail whether human tissue mast cells are an important source of IL-17A.
MATERIALS AND METHODS

Ethics

Human tonsils were obtained as leftover material after tonsillectomies, as approved by the Medical Ethical Committee of the Slotervaart Hospital, Amsterdam. Human synovial biopsies were obtained after written informed consent, as approved by the Medical Ethics Committee of the Academic Medical Center.

Cells isolation and flow cytometry analysis

Tonsil tissue was mechanically disrupted using the Stomacher 80 Biomaster (Seward). Mononuclear cells were isolated with Ficoll-Paque Plus (GE Healthcare). Synovial tissue samples were obtained from inflamed joints by arthroscopy, as described previously [43]. Ex vivo the tissue was digested using Liberase TM (Roche) and DNAseI (Roche) to obtain a single-cell suspension. For phenotypic analyses by flow cytometry data were collected on FACS Canto II instrument (BD Biosciences) and analyzed with FlowJo software (TreeStar). Tonsil mononuclear cell samples were enriched for c-kit (Miltenyi). Mast cells and T cells were further purified by two consecutive rounds of flow sorting with FACS ARIA II (BD Biosciences) to obtain > 99.5% purity.

Reagents

Antibodies against human proteins: IL-17A, (AF-317-NA and MAB3171, R&D), RORC (14-6988, eBioscience), IL-17F (AF1335, R&D), TNF-α (AB-210-NA, R&D), IL-22 (AF782, R&D), IL-23p19 (14-7238-80 ebioscience), β-actin (sc-1616, Santa Cruz), tubulin (T9026, Sigma), FcεR1(BD), CD4(BD), CD3(BD) c-kit(BD). Antibody against murine IL-17A (AF-421-NA, R&D). Antibody against GFP (14-6674 eBioscience). ELISA: IL-17A(eBio64CAP17 and eBio64DEC17) Stimuli: PMA (10 ng/ml; Sigma) and ionomycin (500 nM; Merck), LPS (10 ng/ml, Sigma), IL-23 (10 ng/ml, R&D), IL-1β (10 ng/ml, R&D). Recombinant protein: hrIL-17A (317-ILB, R&D), hrIL-17A-his (ab166882, Abcam), hrTNF-α (PHC3015, Life Technologies), rhIL-22 (200-22 Peprotech) and rhIL-23 (200-23 Peprotech).

Establishment of cell lines

Primary mast cells were analyzed directly or were cultured on a monolayer of fibroblast-like synoviocytes (FLS) with addition of 100 ng/ml stem cell factor (SCF)(Peprotech [44]). The LAD2 cell line was a gift from Dr. Kirshenbaum (National Institutes of Health, Bethesda, MD). Primary mast cells and LAD2 cells were cultured in StemPro-34 (Life Technologies) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin, and 100 ng/ml SCF [45].

Quantitative PCR analysis

Ex vivo purified c-kithigh/FcεR1+ mast cells and CD4+ T cells were analyzed directly or after stimulation for 2-10 hours. mRNA was extracted with nucleospin XS columns (Machery-Nagel) and reverse transcribed with cDNA Synthesis Kit (Fermentas). mRNA from isolated
synovial tissue cells was amplified with SMARTer PICO kit following manufacturer’s instructions (634928, 639207, Clontech). cDNA was used as template for real-time PCR analysis on a StepOnePlus System (Applied Biosystems). Expression of the target gene was normalized to the expression of the housekeeping gene GAPDH. The following gene expression assays (TaqMan) were used IL-17A (Hs00174383_m1), IL-17F (Hs00369400_m1), IL-17B (Hs00975262_m1), IL-17C (Hs00171163_m1), IL-17D (Hs00370528_m1), IL17E/IL25 (Hs03044841_m1), CMA1 (Hs00156558_m1), TNF (Hs00174128_m1), IL-6 (Hs00174131_m1), RORC (Hs01076122_m1), CD3d (Hs00174158_m1), KIT (Hs00174029_m1), IL-17RA (Hs01064648_m1), IL-17RC (Hs00994305_m1), IL-17RB (Hs00218889_m1) and GAPDH (4310884E) (all Life technologies).

**Immunoblot analysis**

For immunoblot analysis equivalent numbers of purified mast cells and CD4+T cells were lysed in Laemmli buffer supplemented with β-mercaptoethanol and denatured at 95°C for 5 minutes. Lysates were resolved by electrophoresis and were transferred onto PVDF membranes. Membranes were washed in tris buffered saline (TBS) (pH 8.0) containing 0.05% Tween-20 (TBST), blocked with milk, and incubated with primary antibodies. IRDye800- or IRDye680- (LI-COR Biosciences) or horse radish peroxidase (HRP)-conjugated secondary antibodies (DAKO) for visualization on the Odyssey (LI-COR Biosciences) or after addition of substrate (Lumi-Light, Roche) on the LAS4000 (GE-Healthcare). Protein quantifications were performed on the data acquired with the Odyssey, chosen for its excellent linear characteristics between fluorescence intensity and protein quantity. Image studio software (LI-COR Biosciences) was used to calculate total fluorescence intensity of the bands.

**IL-17A uptake and release**

LAD2 mast cells were incubated overnight with 5 µg/ml, or for 2 to 4 hours with 25 µg/ml of recombinant protein. After incubation cells were washed with medium four times, including a wash step with acetic acid solution (pH=2.9), in order to remove membrane-bound protein. The dynaminI GTPase inhibitor, tetradecyltrimethylammonium bromide (MiTMAB) (324411, Merck) was used to block dynaminII-mediated endocytosis. LAD2 mast cells were pre-treated for 40 minutes with trypsin 0.25% in order to cleave all membrane proteins and after thorough washing uptake was assessed. Released IL-17 was assessed, after incubation with rhIL-17A, consequent washings and re-suspension in fresh medium. Release was inhibited by lowering the incubation temperature and by adding sodium cromoglycate (1 mM, C0339, Sigma). Activity of released IL-17A was measured by production of IL-6 by NIH-3T3 mouse embryonic fibroblasts. Conditioned media were created by resting LAD2 cells, that were previously incubated with rhIL-17A, at a density of 2*10^6 cells/ml for 24 hours in fresh medium. Specific neutralizing antibodies against IL-17A (AF-317-NA, R&D) or isotype-matched irrelevant antibodies (AB-108-C, R&D) were used at 1 µg/ml final concentration. Conditioned media were diluted 1:1 in DMEM and supplemented with fetal bovine serum 10% final concentration and TNF-α (PHC3015, Life Technologies) 2ng/ml final concentration. 10^5 cells (NIH-3T3-MEF’s) were serum starved for 24 hours, after which the media were
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replaced by diluted conditioned media. After 24 hours the media were collected and were tested for murine IL-6 by ELISA (DY406 R&D).

**Immunofluorescence**

Five-micrometer sections of paraffin-embedded tonsil tissue were deparaffinized and rehydrated. After antigen retrieval by heating in 0.5 M citrate buffer (pH 6), the sections were blocked with 10% donkey serum. Incubation with mouse-anti–mast cell tryptase (clone AA1; Abcam) and goat-anti-IL-17 (AF-317-NA, R&D Systems), followed by incubation with AF488 donkey-anti-goat and AF555 donkey-anti-mouse antibodies. The protocol could be extended with an incubation with rabbit monoclonal antibodies from the endosomal trafficking kit (#9765, Cell Signaling) or biotinilated-anti-LAMP1 (MAB4800, R&D). AF647 donkey-anti-rabbit or streptavidin antibodies were used for detection. Lipid membrane was stained with CellMask™ Deep Red Plasma membrane Stain (C10046, Life Technologies). Slides were mounted with ProlongGold (Life technologies) and analyzed on a confocal microscope (SP-8 X, Leica DMRA). Confocal stacks were deconvoluted with Huygens software (Scientific Volume Imaging) and rendered to high quality, high definition, maximum-intensity projections of one or two consecutive images.

**Statistics**

PrismV5 software (GraphPad) was used for statistic testing. A p-value of 0.05 or lower was considered statistical significant.

**RESULTS**

**Primary human tissue mast cells contain IL-17A protein but lack its transcriptional machinery.**

The evidence that IL-17A containing mast cells and neutrophils actively produce IL-17A is scarce [22] and in contradiction with murine data [38–42]. To address this discrepancy, we first investigated the capacity of primary human tissue mast cells to produce IL-17 family cytokines de novo. We analyzed mRNA expression of Il17a, Il17f, and Rorc in highly purified human tonsillar mast cells and, as control, CD4+ T lymphocytes isolated from the same source (purity of both populations > 99.5% by double flow-sorting). IL-17A transcripts were undetectable in mast cells and very low in CD4+ T cells when analyzed directly ex vivo (Fig. 1A). Mast cells also failed to express significant levels of IL-17A mRNA after ex vivo stimulation with phorbol myristate acetate (PMA)/ionomycin for 10 hours, whereas the control population of CD4+ T cells from the same tissue clearly did express these transcripts (Fig. 1A). This finding was confirmed by a series of additional experiments. Firstly, IL-17F expression showed an identical profile as IL-17A (Fig. 1B). Secondly, other stimulatory conditions, including a combination of IL-23 and IL-1β which has been shown to potentiate IL-17A production by T cells and blood-derived mast cells [22,46], also failed to induce IL-17A mRNA in human tonsillar mast cells (data not shown). Thirdly, human mast cells isolated from a different target organ, synovial tissue, did also not express IL-17A or IL-17F mRNA,
Figure 1. Isolated tissue mast cells do not produce IL-17A, but do store IL-17A in granules. qPCR analysis of A, IL-17A B, IL-17F and C, RORC expression in sorted tonsil mast cells and CD4+ T cells ex vivo or after stimulation with PMA/ionomycin D, Protein content of sorted mast cells and CD4+ T cells, ex vivo western blot for IL-17A and RORC. E, IL-17A presents in granule-like structure in a single mast cell on a tonsil section, mast cell tryptase (red), IL-17A (green) and DAPI (cyan/blue). Projection of confocal z-stack. F, Subcellular colocalization of IL-17A with LAMP1 (cyan/blue) and G, EEA1 (cyan/blue).
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either directly ex vivo (Suppl. Fig. 1A) or after stimulation (Suppl. Fig. 1B). Finally, the mRNA expression of RORC, the transcription factor controlling IL-17A expression, was very low to absent in tonsillar mast cells compared to paired CD4+ T cells (Fig. 1C).

Since previous studies in human defined IL-17A-positive mast cells by immunostaining [22–36], which may be sensitive to biases such as cross-reactivity of detection antibodies or visualization of IL-17A bound to receptors on the cell surface, we next verified the presence of IL-17A protein in isolated human tonsillar mast cells by immunoblotting and confocal microscopy. Immunoblotting of mast cell protein extracts with the goat polyclonal anti-human IL-17A antibody widely used in the previously mentioned histological studies as well as with a monoclonal mouse anti-human IL-17A antibody revealed a 17 kDa protein migrating together with recombinant IL-17A (Fig. 1D and Suppl. Fig. 2A) and with a similar band in a protein extract of paired tonsillar CD4+ T cells (Fig. 1D). Strikingly, protein expression of RORC, the transcriptional factor indispensable for IL-17A expression, was detected in tonsillar CD4+ T lymphocytes but not in mast cells (Fig. 1D), confirming that mast cells lack active transcription machinery for IL-17A expression.

In order to formally demonstrate that IL-17A protein is detected inside mast cells and not merely on membrane-bound IL-17 receptors, additional evaluation of IL-17A in human tonsillar tissue mast cells demonstrated a granular staining pattern throughout the cytoplasm of the cell (Fig. 1E). Double staining of the IL-17A-positive granules in tonsil sections showed partial intracellular co-localization with mast cell tryptase (MCT) as well as with lysosomal associated membrane protein 1 (LAMP1), a marker for lysosomal storage granules and mature lysosomes (Fig. 1F). Of interest, we also detected co-localization of IL-17A with early endosome antigen 1 (EEA1), a specific marker for early endosomes (Fig. 1G), suggesting a role for endocytosis in the IL-17A biology in mast cells.

Human mast cells can engulf exogenous IL-17A protein

Based on the presence of IL-17A protein but not its transcriptional machinery and on the co-localization of IL-17A with EEA1 in human tissue mast cells, we hypothesized that mast cells are capable of engulfing and storing exogenous IL-17A protein. We used the human mast cell line LAD2, which does not contain IL-17A protein in homeostatic culture conditions and does not produce IL-17A upon stimulation with LPS or PMA/ionomycin (data not shown), to test this hypothesis. In contrast to the standard culture conditions, IL-17A was clearly present in LAD2 cell lysate after incubation of LAD2 cells with recombinant IL-17A overnight at 37°C followed by a thorough acid wash to remove membrane-bound protein (Fig. 2A), suggesting that this mast cell line can take up exogenous IL-17A. The exogenous origin of IL-17A in these experiments was confirmed by the fact that incubation with recombinant GFP-tagged as well as 6xhistidine-tagged IL-17A protein, but not with GFP alone lead to detection of a granular intracellular signal for the tagged proteins (Fig. 2B, 2C). Quantification by imaging flow cytometry showed that not only LAD2 cells but also ex vivo tonsillar mast cells are capable of IL-17A uptake (Fig. 2D). The engulfed exogenous IL-17A was localized in intracellular organelles as visualized by lipid membrane staining after mechanical disruption
Figure 2. Mast cells engulf IL-17A protein. A, Exogenously administered IL-17A retains in mast cells after overnight incubation and removal of all extracellular IL-17A by acid washings. B, exogenously administered IL-17A-GFP (green) but not GFP is detected in a granular pattern after incubation. Wide-field microscopy, C-kit (red), nuclei are stained with DAPI (blue). C, Imaging flow cytometry revealed a similar pattern exogenously administered IL-17A-GFP and IL-17A-6xhis. 6xhis was visualized using a labeled anti-6xhis antibody. C-kit (red) and DAPI (blue) D, Quantification of uptake of exogenous IL-17A in LAD2 cells and primary mast cells from tonsil. Criteria for positive cells were computationally defined. E, Detection of vesicle shaped organelles after mechanical disruption of cells that have been incubated with IL-17-GFP. The organelles lipid membranes are shown in red. F, Colocalization of IL-17A-GFP (green) with LAMP1 (red). Cytospinned LAD2 cells after administration of exogenous IL-17A-GFP. G, No colocalization of IL-17A-GFP with lysotracker in living LAD2 cells.

of the mast cells (Fig. 2E). Similar to our in vivo observations (Fig. 1F), double staining of intact cultured cells indicated that exogenous IL-17A engulfed by mast cells was mainly found in LAMP1-positive organelles (Fig. 2F). These IL-17A-positive organelles are not mature
lysosomes as they were negative for LysoTracker (Fig. 2G). Collectively, these ex vivo/in vitro experiments indicate that human mast cells are able to engulf exogenous IL-17A and store it in intracellular granules, similar to what is seen in human tissues in vivo.

**Specific internalization of IL-17A by receptor-mediated endocytosis**

We assessed the potential involvement of the motor GTPase dynaminII, which is important in uptake processes like receptor-mediated endocytosis or fluid phase endocytosis. The dynaminII inhibitor, Mitmab, induced a dose-dependent decrease of exogenous IL-17A uptake by LAD2 cells (IC$_{50}$ ~30 µM) as measured by quantification of western blot after thoroughly washing cells with PBS and acid washings (Fig. 3A) Mitmab inhibits growth and induces cell death in various cancer cell lines [47], we found that LAD2 cell viability was not effected for concentrations upto 50µM of Mitmab (data not shown). Dynamin II can be involved in fluid phase endocytosis or in uptake with help of a membrane receptor (receptor mediated endocytosis). We first compared the uptake of IL-17A-GFP simultaneous with dextran (10 kDa), a prototypic molecule that is exclusively internalized by fluid-phase-endocytosis (Fig. 3B). Secondly we compared transferrin, a prototypic molecule that is internalized by receptor-mediated-endocytosis (Fig. 3C). For dextran, even at double molar concentration, we did not observe internalization, at that moment IL-17A-GFP was already clearly visible in the cells (Fig. 3B). For transferrin we observed similar uptake kinetics as with IL-17A-GFP (Fig. 3C). As an alternative approach to differentiate between fluid phase- and receptor mediated endocytosis, we have 'shaved' all the protein from the cell membrane, by enzymatic treatment. Cells that were treated were no longer able to internalize IL-17A (Fig. 3D). As these data suggest that the internalization of IL-17A is most likely mediated by a membrane receptor. We further assessed the specificity of uptake by incubation of LAD2 cells with recombinant IL-17A, IL-17F, TNF-a, IL-22 and IL-23 at either 4°C or 37°C. At 4°C, a temperature where active processes such as receptor-mediated endocytosis are blocked, we did observe low signals for all proteins tested, reflecting the background in this experiment. At 37°C, however, we did observe a marked and significant uptake of IL-17A, reduced but significant uptake of IL-17F, but no uptake of TNF-a, IL-22 or IL-23 (Fig. 3E). Hypothesizing that known IL-17 receptors may be involved in this process, we first characterized the expression of the receptors for IL-17A, such as IL-17RA and IL-17RC, on the LAD2 cells. LAD2 cells expressed IL-17RA, but not IL-17RC, as transcript and cell surface protein (Fig. 3F,G). Similarly, primary tonsillar mast cells expressed IL-17RA, with only a subpopulation co-expressing IL-17RC (data not shown). Neutralizing IL-17RA with blocking antibodies did not affect uptake of recombinant IL-17A by LAD2 cells in vitro (Fig. 3H). In line with these findings in human, IL-17A protein was found in purified murine peritoneal mast cells in wild type as well as in IL-17RA knock-out mice [3I]. Collectively, these data indicate that IL-17A uptake by human mast cells is an active, specific, and presumably receptor-mediated process, although IL-17RA and IL-17RC-independent.
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**Figure 3. IL-17A uptake via receptor mediated endocytosis.**

A, inhibition of Dynamin II-GTPase results in decrease of uptake, quantification by western blot (N=5)  B, Dextran, a prototypic molecule that can only be taken up by fluid-phase endocytosis, is not taken up where IL17-GFP is already detected. Still from time-lapse movie C, Transferrin, a prototypic molecule that is taken up by receptor mediated endocytosis, is taken up by LAD2 cells together with IL-17A-GFP, though it is sorted in different granules. Still from time-lapse movie. D, Uptake is inhibited by enzymatic cleavage of membrane proteins. E, Batches of LAD2 cells were incubated either at 4°C or at 37°C for 3.5 hours with 25ug/ml recombinant IL-17A, IL-17F, TNFα, IL-22 and IL-23. Cells were washed, including a step with acetic acid, and total lysates were blotted for the respective target protein and a household protein. Quantification of the band intensity ratio is represented (N=5). A picture of a representative membrane is shown under the quantification. F, qPCR analysis of IL-17 receptors on LAD2 cells (n.d. not detected) G, Protein analysis by flow cytometry of IL-17 receptors on LAD2 cells. Grey fill shows intensities for unstained cells, dotted line for cells stained with IL-17RC and the solid line for cells stained with IL-17RA. H, Batches of LAD2 cells were incubated with exogenous IL-17A, one batch received no antibodies, one batch received antibodies that neutralize binding of IL-17A to IL-17RA, one batch received irrelevant antibodies with the same isotype. No modulation was observed by the specific antibody. I, Purified cells from mouse peritoneal lavages show IL-17A protein in mast cells in wild type and IL-17RA-/-.. Simultaneously purified control cells (CD11+) did not contain IL-17A.
Human mast cells are able to release the internalized recombinant IL-17A as a bio-active cytokine

The capacity of mast cells to capture exogenous IL-17 may be relevant to quench IL-17A either to neutralize excess protein during tissue inflammation or, alternatively, to quickly release pre-stored bioactive IL-17A upon an acute insult. We did not find evidence for active degradation of IL-17A, firstly internalized exogenous IL-17A did not co-localize with lysotracker (Fig. 2G) in LAD2 cells and when primary human mast cells were isolated from tonsils and cultured for 3 weeks ex vivo, IL-17A protein was still abundantly detectable in intra-cellular granules (Fig. 4A), indicating that the protein is not efficiently degraded. We next assessed whether intracellularly stored exogenous IL-17A can be actively released in the culture supernatant by mast cells. After feeding LAD2 cells with recombinant IL-17A and extensive washing as described above, the human mast cells released pre-stored IL-17A in the culture supernatant as measured by ELISA by an active process, as evidenced by reduction of release at 4°C versus 37°C (Fig. 4B). Release of IL-17A by the LAD2 cells was also significantly inhibited by sodium cromoglycate (cromolyn), a stabilizer of degranulation (Fig. 4C).

To assess if the IL-17A that is released by the mast cells still has biological activity, we tested various conditioned mast cell media on the IL-17A responsive NIH-3T3 mouse embryonic fibroblasts (MEF), a cell line that is commonly used to test the activity of recombinant human IL-17A [48]. Because the levels of IL-17A in the conditioned medium were typically less than 1 ng/ml, we made use of a suboptimal dose of TNF-a to increase the sensitivity of the cell line to IL-17A [49]. Using IL-6 secretion by the NIH-3T3-MEFs as a readout, we demonstrated a clear response to supernatant of IL-17A fed mast cells compared to unfed mast cells (Fig. 4D). This response was induced by IL-17A released in the conditioned media as the IL-6 secretion by the responder fibroblast cell line could be blocked by the addition of specific neutralizing antibodies against IL-17A but not with irrelevant control antibodies (Fig. 4D). Taken together these experiments indicate that the exogenous pre-stored IL-17A can be released by the mast cells and is biologically active.

DISCUSSION
Numerous studies in rodents and humans have demonstrated that IL-17A, a key pro-inflammatory cytokine in different types of chronic tissue inflammation, is not exclusively produced by classical Th17 cells but also by other lymphocytes, including Tc17, γδ-T cells, and subsets of innate lymphoid cells [50–52]. To what extent non-lymphocytic immune cells such as mast cells and neutrophils can produce IL-17A remains highly debated. IL-17A+ mast cells are omnipresent as a dominant population amongst IL-17A-expressing cells in human tissues [22–36]. The expression of IL-17A mRNA, however, is conflictingly reported. First, two recent studies present contradicting conclusions regarding to the production of IL-17A mRNA expression in the skin: a RT-PCR based approach detected no IL-17A transcripts [53] while an approach with flow based hybridization clearly detects expression [54]. Additionally, two studies were able to demonstrate transcription of IL-17A mRNA by human mast cell lines:
Figure 4. Endocytozed IL-17A can be actively secreted and is bioactive. A, Primary mast cells isolated from tonsil were followed in vitro for three weeks. A clear signal for IL-17A was detected, suggesting a long half-life of the protein within the mast cell, B, Spontaneous exocytosis could be modulated by temperature and C, by the mast cell stabilizer sodium cromoglycate (cromolyn). Levels of IL-17A were measured in the supernatant by ELISA D, The activity of released IL-17A was assessed by adding conditioned media to NIH-3T3-MEFs. Control conditioned medium was created by overnight incubation of LAD2 cells in fresh medium. Alternatively, LAD2 cells that were previously loaded with exogenous IL-17A were left to exocytose the IL-17A in fresh medium overnight (Conditioned medium exocytosed IL-17A). Specific antibodies neutralizing IL-17A were used to define the contribution of IL-17A to the production of IL-6 by fibroblasts. Isotype matched irrelevant antibodies did not modulate IL-6 production. One representative experiment was depicted with >5 technical replicates. Medians from four independent experiments are in line with the representative experiment one study reported RORC-dependent expression of IL-17A mRNA in CD34+ derived human mast cells [22], and the other study reported aryl hydrocarbon receptor-dependent, RORC-independent expression of IL-17A by the human cell lines LAD2 and HMC [55]. As it remains unclear to what extent these cell lines and the in vitro stimulation protocols reflect what is happening in the inflamed human tissues, we aimed to assess IL-17 mRNA expression directly in highly purified (less than 0.5% of contamination) human tissue mast cells using specific RT-PCR methodology. In contrast to paired memory T-cell controls, these tissue mast cells did not express significant mRNA levels of IL-17A, other IL-17 family members, or
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RORC, either directly ex vivo or after in vitro stimulation. Investigating the reported RORC-independent mechanism [55], we were unable to reproduce the finding of IL-17A mRNA transcription by the LAD cell line after stimulation with either PMA/ionomycin, LPS, or IL-1b/IL-23 or the aryl hydrocarbon receptor agonist FICZ (including experiments following the exact protocol as in ref 55). Although we cannot formally exclude that human mast cells can produce IL-17A during early stages of differentiation as suggested by the CD34+ derived mast cell data [22], our data demonstrate a striking discrepancy between the abundant intracellular presence of IL-17A protein in human tissue mast cells that we confirmed using a variety of technologies, and the absence of the transcriptional machinery for this protein. In analogy with the conflicting results on IL-17A+ mast cells, equal debates are ongoing on IL-17A+ neutrophils. A landmark paper provided evidence that neutrophils produce IL-17A+ and that this is clinically relevant for clearing fungal infection [56]. However, recent studies are not able to repeat their findings with human samples [57] and a study especially aimed to prove or disprove clinical relevance of IL-17A produced by neutrophils in candida infection in mice turned out negative [42].

Detailed microscopic study of IL-17A protein within mast cells revealed its localization in intracellular storage granules and trafficking through early endosomes, suggesting mast cell may acquire and store exogenous IL-17A. Interestingly, it has been shown previously that TNF-a produced by mast cells is not stored immediately in granules but is consecutively transcribed, translated, directed towards the canonical cytokine excretion pathway, expressed on the plasma membrane, and re-endocytosed to be stored in granules [58]. Exploring if a similar endocytosis and storage mechanism despite the absence of endogenous production may explain the intracellular presence of IL-17A protein in absence of transcriptional activity, we were able to demonstrate by Western blot, confocal microscopy, and imaging flow cytometry that human mast cell lines as well as primary human tissue mast cells are capable of engulfing exogenous IL-17A. The recombinant exogenous IL-17A was subsequently stored in LAMP1 positive cytoplasmic granules resembling the IL-17A containing vesicles that we observed in vivo. This phenomenon, where human mast cells capture and store exogenous proteins from the environment, has, to our knowledge, not been described before for cytokines. However, a study of mast cells in rat mammary tumors performed more than 25 years ago reported that these cells were able to collect and accumulate prolactin in their cytoplasmic granules [59], supporting the existence of such a phenomenon in mast cells.

The mechanism underlying this unique feature remains incompletely understood. The uptake of prolactin by rat mast cells was described to be time-, energy-, and temperature-dependent and was suggested to be mediated by the prolactin receptor [59]. In line with these data, we provided evidence that IL-17A uptake by human mast cells is an active process dependent on DynaminII-GTPase. Further, we have excluded fluid phase endocytosis and we have shown that one or more membrane protein are indispensable for the uptake process. Taken together, these data strongly suggest involvement of the well-studied process, receptor mediated uptake. This process is highly specific for a ligand, and indeed we have not observed uptake of control cytokines such as TNF-a, IL-22 and IL-23.
Unfortunately, we have not identified an indispensable receptor. Neither IL-17RA nor IL17RC, which are the high affinity receptors for IL-17A [60], appear to be implicated in this process. IL-17RA is expressed on human mast cells, but inhibition by either monoclonal antibodies for human mast cells or by genetic deletion for mouse mast cells did not influence the uptake. IL-17RC is not expressed by human mast cells. The involvement of other putative IL-17A-binding receptors, in particular IL-17RD [61], remains to be formally established.

Uptake of exogenous IL-17A by mast cells could serve different functions in the regulation of IL-17A-mediated inflammatory responses. Human tissue mast cells could act as a sink by quenching and neutralizing excess IL-17A during tissue inflammation. Intriguingly, however, our in vitro assays indicated that exogenous IL-17A protein was not directed towards mature lysosomes, and ex vivo culture of primary tissue mast cells demonstrated that IL-17A remained stored in the cytoplasmic granules for several weeks. Consistent with this notion that IL-17A is not degraded after uptake by human mast cells, we were able to demonstrate that stored exogenous IL-17 can actively be released into the supernatant in a structurally and functionally intact form. Again, this is consistent with the notion that rat mast cells can excrete stored exogenous prolactin [59]. Although it remains to be firmly established which triggers specifically drive this release, our data point towards a scenario where mast cells could contribute to host defense and/or to tissue inflammation by rapid release of pre-formed bioactive IL-17A. Accordingly, we propose that stored exogenous IL-17A complements the other pre-stored inflammatory mediators to enable mast cells to function as sentinel cells in various tissues.

This novel mechanism of exogenous IL-17A protein uptake, storage, and release by primary human mast cells raises a series of additional questions. Firstly, extensive investigations are needed to assess if this mechanism is highly specific for IL-17A or may apply to a larger panel of cytokines and mediators. Our experiments indicate at least some specificity, as TNF-a as well as IL-22 and IL-23 were not taken up, but it should be noticed that we also observed low-level uptake of IL-17F. Secondly, the current study focusses on mast cells, but it remains unknown if this mechanism applies to all mast cells and if it may also apply to other cell types. As to mast cell heterogeneity, we have observed similar characteristics of uptake and similar levels of tryptase and chymase expression in mast cells isolated from tonsil as from synovial tissue. We have not assessed heterogeneity within a tissue source. As to other cells besides mast cells, neutrophils are of particular relevance as they also show co-localization of IL-17A protein by immunostaining in human tissue samples, but de novo IL-17A production by in these cells is debated [57]. Thirdly and finally, a key question is the relative contribution of mast cell pre-stored IL-17A versus de novo synthesized IL-17A by other cell types in vivo in different physiological and pathological conditions. Unraveling the exact molecular mechanisms of this novel level of regulation of IL-17A, may enable new ways of targeting the IL-17A axis in a variety of human conditions.
AUTHOR CONTRIBUTIONS STATEMENT
TN, NY, DB designed the study and wrote the paper. TN, IB, SC, NY, EL performed experiments and analyzed data. SC, JS, EM, EL, JDC provided crucial technical assistance. All authors have reviewed the final version of the paper.

ACKNOWLEDGEMENT
We thank Dave Speijer and Kristine Germar for intellectual discussions and revision of the manuscript.

CONFLICT OF INTEREST DISCLOSURE
None of the author disclosed competing interests for this study.

FUNDING
Troy Noordenbos is supported by an unrestricted grant (PHD-scholarship) awarded by the Academic Medical Center. Dominique Baeten is supported by a VICI grant of the Netherlands Scientific Organization (NWO), a Consolidator grant from the European Research Council (ERC), and grants of the Dutch Arthritis Foundation (Reumafonds).
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Supplementary material 1. No IL-17 gene expression in human synovial tissue mast cells. A, Mast cells isolated from human synovial tissue did not express IL-17A, nor other IL-17 family members ex vivo. B, Stimulation of isolated human synovial tissue mast cells with LPS, IL-23 or a combination did not induce expression of IL-17A or IL-17F.
Supplementary Material 2. IL-17 protein expression in human and mouse mast cells. A, Lysates of sorted mast cells, CD3+ T cells and CD3+/CD4+ T cells stained with mouse monoclonal antibody against IL-17A and goat polyclonal antibody against IL-17A. Pictures from a full membrane from a representative tonsil donor B, LAD2 cell line. Competition for the putative binding sites on IL-17A for heparan sulphate glycosaminoglycans by addition of heparin to the medium did not decrease the capacity of mast cells to take up IL-17A.